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13. ABSTRACT ***(Maximum 200 Words)***

Recent evidence suggests one reason for the rise in breast cancer incidence is increased exposure to and bioaccumulation of environmental pollutants. Work from the PI's laboratory has demonstrated that human breast tumor cell lines and primary breast tumor tissue constitutively express high levels of functional nuclear NF-κB/Rel activity, in contrast to untransformed breast epithelial cells or mammary tissue. Experiments are proposed using cell lines in culture, primary tissue and a transgenic mouse model to test the role of nuclear NF-κB/Rel activity in the etiology of breast cancer. The results of these studies will provide important information on the potential role of NF-κB/Rel factor overexpression in the etiology of breast disease. Rel factors represent an important link between environmental factors and the increased incidence of breast cancer. The different patterns of Rel factor expression in various tumors suggest the possiblity that NF-κB/Rel factor represents a new class of potential marker(s) for analysis of progression of breast disease. Importantly, since Rel factor activity is sensitive to treatment with a number of anti-oxidants, demonstration that NF-κB/Rel factors play a role in the etiology of breast cancer would provide a new therapeutic target for the treatment of breast disease.

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INTRODUCTION

The incidence of breast cancer has been steadily increasing over the past 50 years, and is now one of the leading causes of death among American women between the ages of 40-55 (23). In an attempt to find the reasons for this steady increase in incidence, both genetic and environmental factors are being studied. Attention has recently focused on the mechanism by which increased exposure to and bioaccumulation of these pollutants might have an etiologic role in breast cancer (13,20,37,59). The polycyclic aromatic hydrocarbons (PAHs) such as 7,12-dimethylbenz(*a*)-anthracene (DMBA) are specifically of interest (37). The most proximal event in PAH tumorigenesis is the binding of the chemicals to a cytosolic aromatic hydrocarbon receptor (AhR) (10,18,47). The receptor-ligand complex is translocated to the nucleus where it can bind to and alter the transcriptional level of DNA that has AhR-responsive elements. One battery of enzymes whose transcriptional induction is a hallmark of DMBA and other PAH exposure is the Phase I cytochrome P450 enzymes (9,32,37,39,61). These enzymes aid in the oxidative metabolism of both endogenous substances such as steroids, as well as in the breakdown of exogenous substances such as drugs, chemical carcinogens, and environmental pollutants. The products formed by this oxidative metabolism are often reactive oxygen intermediates (61). The potential for increased levels of oxidative stress within the cell resultant from exposure to environmental carcinogens leads us to hypothesize that this might activate expression of the NF-κB/Rel family of transcription factors. This family of factors, which regulates transcription of multiple genes including those involved in the regulation of cell proliferation, such as the c-*myc* oncogene implicated in neoplastic transformation (17,27,31), has been found to be sensitive to the cellular redox state (rev. in 1). In agreement with this model, in preliminary experiments we have found that malignant breast cancer cell lines and primary breast cancer tissue express significant levels of constitutive nuclear NF-κB/Rel activity. The constitutive expression of this factor suggests that NF-κB/Rel may promote aberrant proliferation and thus play an early role in the etiology of breast cancer. A brief introduction to NF-κB/Rel, in particular as it relates to this proposal follows.

NF-κB/Rel Family of Transcription Factors

The transcription factor NF-κB was first identified as a protein specific to mature B lymphocytes that interacted with the B site of the kappa light (L) chain gene enhancer (52). Constitutive nuclear NF-κB activity appeared to occur only in mature B lymphocytes. In most non-B cells, inactive NF-κB protein is present sequestered in the cytoplasm with inhibitor proteins termed IκBs (2). Activation of the NF-κB/IκB complex involves phosphorylation and degradation of IκB (8,24), allowing for translocation of active NF-κB complex into the nucleus where it can bind to κB responsive elements. Activation and nuclear localization can be induced by several agents, including oxidative stress (reviewed in references 1,5,22). NF-κB has been implicated in transcriptional regulation of a number of cellular genes involved in control of cell proliferation, adhesion, and in immune and inflammatory responses (1,5,22). These include the oncogene c-*myc*, several genes encoding growth factors or interleukins or their receptors, and adhesion molecules such as E-selectin, ICAM-I, and VCAM-I. We demonstrated that the murine c-*myc* oncogene contains two functional κB sites (16,28). The human c-*myc* gene was found to contain similar κB elements (25).

The biochemical characterization of classical NF-κB determined that it is a heterodimer composed of a 50 kDa (p50) and a 65 kDa (p65) subunit. Cloning and sequencing of p50 and p65 led to the discovery that the binding domains of these factors have homology with an approximate 300 amino acid domain of the v-Rel oncoprotein and was thus termed the Rel homology domain (RHD), hence this family is termed the Rel or NF-κB/Rel family (21,29,45). In addition to c-Rel,

other members of the Rel family have been discovered, including p52 (also called lyt10) (6,41), RelB (46), and the product of the Drosophila *dorsal* gene (55). Rel-related factors bind as hetero- or homodimers that have different activities depending on subunit composition (58). For example, the p65 subunit is able to potently transactivate promoters driven by κB elements (31,48). The c-Rel protein, which appears to function in an element specific fashion, transactivates more moderately (31, 33,35,56). RelB is also a potent transactivator but only functions as a heterodimer (15,46). The overall effect within a cell is determined by the balance of dimers expressed, and is specific to the gene of interest.

Activation of NF-κB involves release from an IκB molecule and transit to the nucleus. Most studies have focused on the predominant IκB species, IκB-α (43). Several groups have demonstrated a role for CK2 in basal phosphorylation of IκB-α (3,34); these findings implicate CK2 in constitutive activation of NF-κB levels. For induction of NF-κB, phosphorylation, ubiquitination and degradation of IκB-α in the cytoplasm leads to its release and subsequent nuclear translocation. The rapid phosphorylation of IκB-α on serine residues 32 and 36 has been shown to correlate with NF-κB induction in a number of cells, e.g., if either of these serine residues are mutated, NF-κB activity is ablated (7). A large, multi-subunit kinase, containing IκB kinase α and β (termed IKKα and IKKβ, respectively), has been implicated in the phosphorylation of these serine residues on IκB-α protein (36,64). Intracellularly, the phosphorylation of these serine residues targets the IκB-α protein for ubiquitination on lysine residues 21 and 22, which marks it for degradation by the proteosome pathway. More recently additional kinases have been implicated in phosphorylation of IKK and thereby IκB-α and activation of NF-κB, including the serine/threonine kinase Akt (42). Our preliminary results indicate malignant breast cell lines and primary tissue constitutive express active Rel factors, including c-Rel and p65. Given the potential important role NF-κB/Rel may play in the control of cell proliferation further studies of these factors in breast cancer were proposed in this application.

BODY AND CONCLUSIONS

Specific Aim 1: Quantitate and compare the levels of the individual subunits of NF-κB/Rel in nuclei of normal and transformed breast cell lines and primary human breast cancer tissue.

a) Work from our laboratory on the analysis of the breast cancer cell lines was completed during the previous year. Our findings, essentially outlined in the previous progress report, have now been accepted for publication in Carcinogenesis.
b,c) We are now focussing on primary human breast cancer tissue specimens. As discussed above, recent work from several laboratories have implicated 3 kinase families in activation of NF-κB. These include the CK2, IKK signalsome, and Akt kinases. Thus, in our recent work we have begun to extend our studies to the analysis of these kinases, in addition to the subunits of NF-κB, as proposed. In our initial analysis, we have analyzed tumor specimens from 6 individual patients (indicated by number code to protect their identity). EMSA for nuclear NF-κB, shown in Fig. 1, indicates variable levels of binding. Four of the specimens displayed higher levels of binding, essentially equivalent to that seen with the DMBA-transformed MCF-10F-derived D3-1 line. These results suggest the presence of activated NF-κB in these 4 specimens (i.e., 6731, 6921, 6712, 6613).

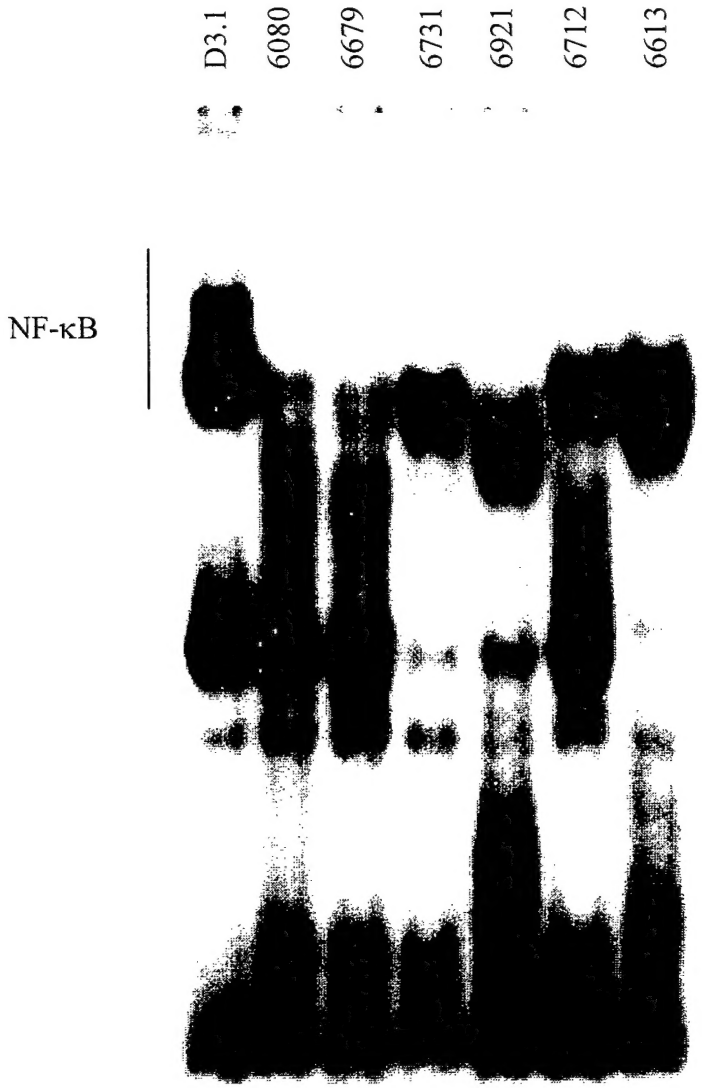

Fig. 1. **NF-κB EMSA of breast cancer patient samples.** Nuclear and cytoplasmic extracts were prepared from breast cancer tumor specimens from 6 individual patients. Patients are indicated by numerical code to protect their identity. Samples (10 ug) of nuclear extracts were subjected to EMSA using the URE NF-κB element from upstream of the c-*myc* promoter, as we have previously described (53). As control, a sample of nuclear extract from the DMBA-transformed human MCF-10F line D3.1 was used a control to localize NF-κB complexes.

To test for contamination with myeloid, macrophage and B cells, EMSA was performed for the PU.1 protein, which is expressed in these cells (Fig. 2). One of the extracts displayed a band of similar size (Patient 6679), while three others had smaller bands, which might be due to partial proteolysis (6613, 6712, 6080). Binding to the TCF-1 T cell specific factor was negative with all of the patient samples (data not shown). From this analysis, it appears that binding of NF-κB in nuclear extracts from patients 6731 and 6921 cannot be explained by mononuclear cell contamination. For patients 6613 and 6712, the contribution of NF-κB binding from contaminating mononuclear cells remains to be determined.

We next assayed for activity of the CK2 and IKK kinases, using wild type (wt) or mutated IκB-α proteins as substrate, using procedures published previously by our collaborators (36,50). Wt IκB-α was phosphorylated by cytoplasmic extracts of all of the patients samples, whereas a mutated form with deletion of the CK2 sites within the PEST domain was not phosphorylated (Fig. 3). Furthermore, addition of apigenin, an inhibitor of CK2 activity, effectively blocked the phosphorylation. When we compared the relative levels of CK2 activity amongst the samples, we noted Patient 6731 displayed a very high level of activity, whereas, it was significantly lower in Patient 6921 (Fig. 3). In addition, high level of CK2 activity was seen with sample 6712. We next assessed basal IKK activity (Fig. 4). We noted that all of the lines displayed constitutive activity with wt IκB-α; the highest level was seen in the 6613 sample. In contrast, no activity was seen with a mutated form containing alanine instead of serine residues at positions 32 and 36, as expected (data not shown).

CONCLUSIONS AND FUTURE DIRECTIONS: Thus, these results indicate that all of the tumor specimens analyzed display constitutive basal IKK activity. Furthermore, the tumor from Patient 6731 displayed elevated levels of CK2 activity and NF-κB binding. In our last year, we propose to extend these studies to include analysis of the three kinases: CK2, IKK and Akt. In addition, we will perform western and antibody supershift analysis to determine subunit expression, as originally proposed. Since mononuclear cells might contain NF-κB confounding interpretation of the results, only samples without evident contamination will be characterized. Additional patient samples are currently being processed for these studies. These studies should provide important information on the mechanism of NF-κB activation in breast cancer patients.

Specific Aim 2: Test the transactivation activity of Rel specific subunits overexpressed in transformed primary tissue in a breast cell line.

As reported in our last Progress Report, we have found an effective means of transfecting untransformed MCF-10F cells. Thus, we have now performed the proposed studies on the functional role of aberrant activation of NF-κB using the c-*myc* gene as target. During the course of these studies, Tian and coworkers (57) reported a physical association between the aromatic hydrocarbon receptor/transcription factor (AhR) and the RelA subunit of NF-κB in murine hepatoma cells. Thus, we tested functional interaction between AhR and RelA in human mammary epithelial cells (HMECs), and found cooperative transactivation of the c-*myc* promoter. A collaboration was arranged with Dr. David Sherr (School of Public Health, Boston University Medical Center), who is a world recognized expert in AhR metabolism and function. Studies were performed to test for AhR and NF-κB subunit association and their ability to regulate expression of *c-myc*. AhR and RelA proteins could be co-precipitated from HMECs. Furthermore, a mechanism involving direct binding via NF-κB elements was implicated in their ability to cooperate to positively transactivate the c-*myc* gene. These findings suggest a new mechanism whereby AhR/NF-κB-dependent activation of the c-*myc* gene can promote proliferation and neoplastic

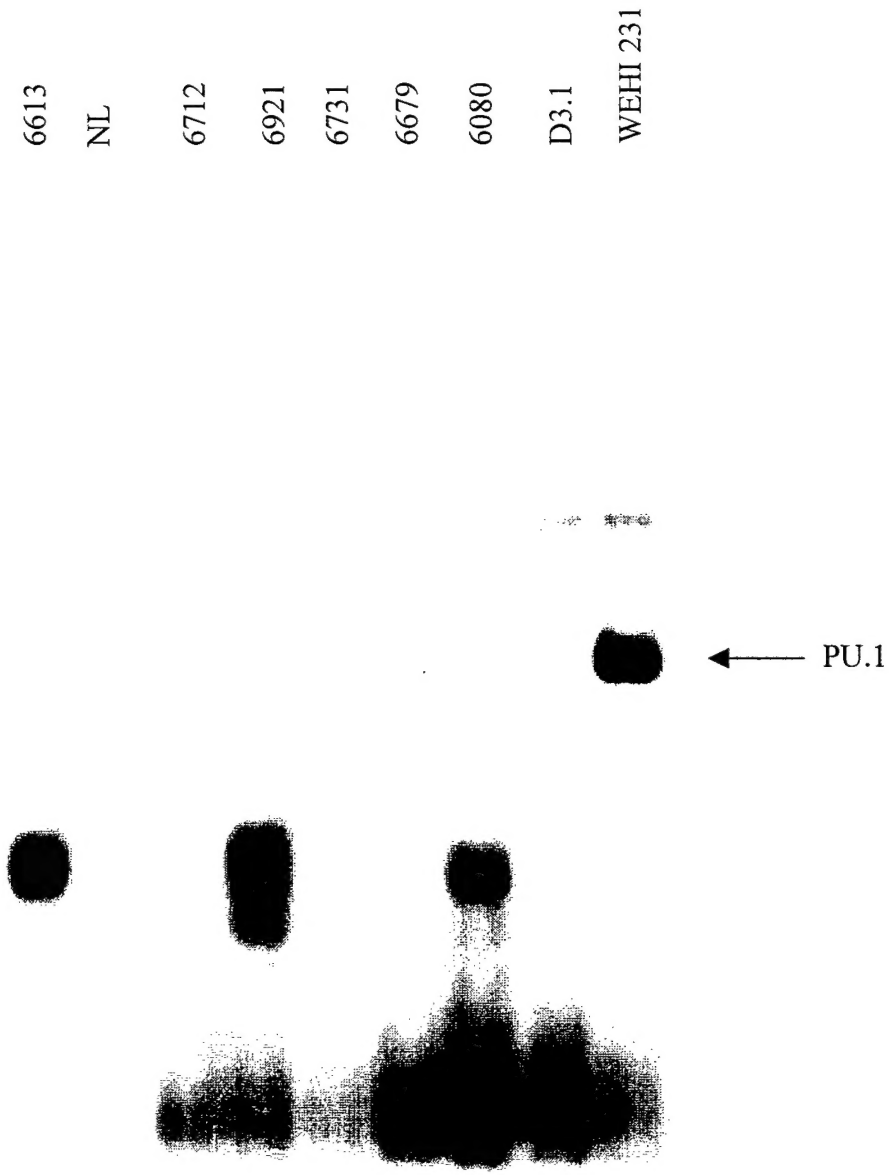

Fig. 2. **PU.1 EMSA of breast cancer patient samples.** Samples (10 ug) of nuclear extracts, prepared in Fig. 1, were subjected to EMSA using the PU.1 element, as we have described previously (53). Samples of extracts from the D3.1 and WEHI 231 B cell lymphoma line were added as negative and positive controls for PU.1 expression, respectively. NL, not loaded.

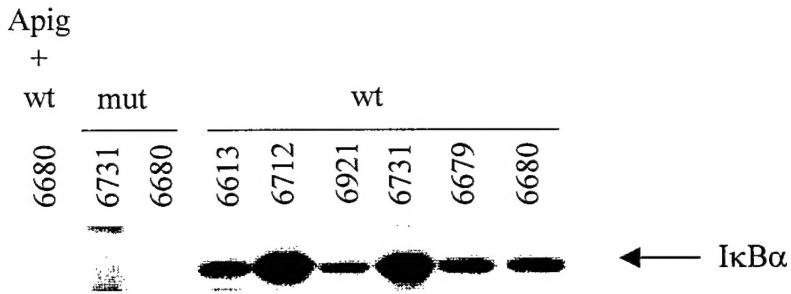

Fig. 3. **CK2 kinase assays of breast cancer patient samples.** Samples (20 ug) of cytoplasmic extracts from the 6 individual patients were subjected to CK2 kinase assays using 5 ug of the following IκB-α-GST fusion proteins as substrate: wild type (wt) or mutated (mut, missing the CK2 sites in the PEST domain). Where indicated 20 uM apigenin (Apig +) was added to the reaction. Reactions were carried out as described previously (50) for 30 min at 30^0C.

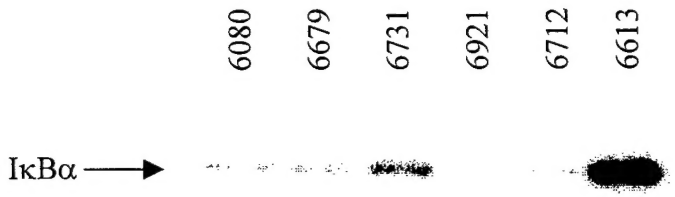

Fig. 4. **IKK kinase assays of breast cancer patient samples.** Samples of cytoplasmic extracts (150 ug) were immunoprecipitated with an antibody against IKK1 (provided by our collaborator F. Mercurio), and subjected to IKK kinase assays using wt IκB-α-GST protein as substrate, as described previously (36).

transformation. A manuscript has been submitted on this work. The findings on activation by NF-κB are discussed in light of this interaction.

AhR and RelA are associated in HMECs-- To assess the association of the RelA subunit of NF-κB with AhR in breast epithelial cells, co-immunoprecipitation studies were performed. Total cell, nuclear, and cytoplasmic extracts were prepared from malignant Hs578T breast cancer cells. Samples of the nuclear (50 μg) and cytoplasmic (100 μg) fractions were then treated with either a goat antibody against AhR (Fig. 5A, lanes 2,3) or a goat IgG fraction, as control (Fig. 5A, lanes 4,5). Immune complexes were isolated using protein A-Sepharose and subjected to electrophoresis, along with a sample of total cell lysate (Fig. 5A, lane 1). The resulting immunoblot was probed with a rabbit polyclonal antibody for expression of the 65 kDa RelA subunit. In the total cell lysate, RelA-specific antibody recognized a protein of the expected molecular weight (65kDa). The AhR antibody, co-precipitated RelA protein from either cytoplasmic or nuclear extracts (Fig. 5A), with somewhat greater amounts seen with the cytoplasmic sample. In contrast, the control goat IgG failed to co-precipitate detectable levels of RelA protein. To confirm this association we performed the inverse experiment of immunoprecipitating nuclear (100 μg protein) or cytoplasmic (200 μg protein) extracts with a rabbit antibody against RelA and then immunoblotting for AhR using a goat antibody. The RelA antibody co-precipitated AhR protein from either cytoplasmic or nuclear extracts (Fig. 5B). In contrast, the control rabbit IgG failed to co-precipitate detectable levels of AhR protein. As seen above, somewhat greater amounts of complexes were seen in the cytoplasm. These findings suggest that endogenous AhR is associated with RelA in both the nucleus and the cytoplasm of Hs578T cells; although, the majority (65-75%) of the complexes are present in the cytoplasm.

We next sought to assess the ability of RelA to associate with the AhR in non-malignant MCF-10F HMECs. To this end, MCF-10F cells were transfected with a vector expressing the *T7-pcDNA3-AhR* vector encoding T7-tagged AhR. Alternatively, cells were transfected with the parental *T7-pcDNA3* DNA, as control. Total cell lysates were prepared, and either immunopreciptated with a RelA-specific antibody or an aliquot run directly on the gel. The resulting immunoblot was probed with a T7 epitope-specific antibody. A protein of the size expected for T7-tagged AhR, i.e. 97-100 kDa, was recognized in total cell extracts from cells transfected with the *T7-pcDNA3-AhR* vector (Fig. 5C, lane 2). Similarly a 97 kDa AhR protein was detected following co-precipitation with RelA-specific antibody (lane 3), whereas no protein was detected following "immunoprecipitation" with control rabbit IgG (lane 1). No anti-T7antibody-reactive protein was detected in extracts from cells transfected with the parental vector (lane 4). Furthermore, AhR protein was not detected in T7-AhR-specific immunoblots of *T7-pcDNA3*-transfected MCF-10F cell extracts precipitated with a p50-specific antibody (data not shown). Similar results were obtained with transfected Hs578T cells (not shown). Overall, these results indicate that the RelA, but not p50, and the AhR are physically associated within Hs578T and MCF-10F cells.

RelA and AhR synergistically activate the c-myc promoter in pre-malignant MCF-10F cells-- To determine whether AhR and NF-κB/Rel can function cooperatively, we examined the effects of *AhR* and *RelA* co-transfection on the c-*myc* promoter. The p1.6Bgl construct, containing –1141 to + 513 bp of the murine c-*myc* promoter linked to a chloramphenicol acetyl transferase (CAT) gene, was used as reporter. Since Dr. Sherr's laboratory has found that endogenous AhR levels decrease in cells as the cultures reach confluence (unpublished observations), all transfections were performed with confluent cultures. In addition, in order to maximize conditions for observing AhR-RelA transcriptional synergy, we first titered the dose of transfected *pEVRF-p65* such that minimal augmentation of reporter activity would be observed. Addition of 1 μg of the *pEVRF-p65*

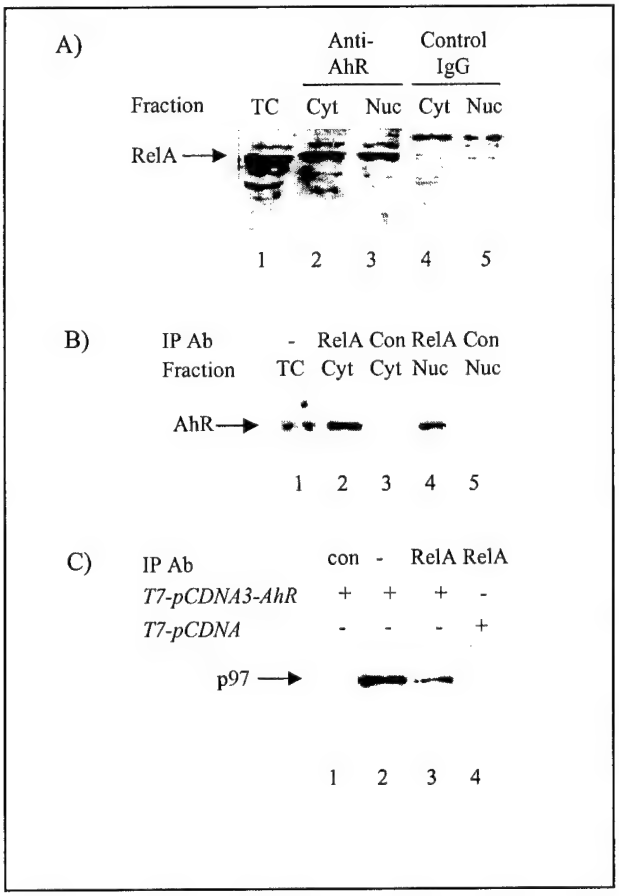

Fig. 5. **AhR and RelA are associated in Hs578T and MCF-10F cells.** A) Cytosolic (100 μg from 1 mg total) or nuclear (50 μg from 128 μg total) proteins from Hs578T cells were immunoprecipitated using 5 μg/ml of either a polyclonal goat anti-AhR antibody (lanes 2,3) or a control goat IgG (lanes 4,5). Of 30 μl of resulting antibody-protein A-Sepharose eluates, 20 μl were subjected to immunoblot analysis with RelA-specific antibody (Cyt: cytosolic immunoprecipitate; Nuc: nuclear immunoprecipitate). Total cell lysate (30 μg protein) was analyzed as a positive control (lane 1) (TC: total cell lysate). The position of the 65 kDa RelA protein is indicated. B) Cytosolic (200 μg from 1.5 mg total) or nuclear (100 μg from 330 μg total) proteins from Hs578T cells were immunoprecipitated using 5 μg/ml of either a polyclonal rabbit RelA-specific antibody (sc-372) (lanes 2,3) or a control rabbit IgG (lanes 4,5). Of 50 μl of resulting antibody-protein A-Sepharose eluates, 30 μl were subjected to immunoblot analysis with polyclonal goat anti-AhR antibody (sc-8088) (Cyt: cytosolic immunoprecipitate; Nuc: nuclear immunoprecipitate). Total cell lysate (40 μg protein) was analyzed as a positive control (lane 1) (TC: total cell lysate). The position of the AhR protein is indicated.
C) Total cell proteins (100 μg) from *T7-pcDNA3-AhR*- or *T7-pcDNA3*-transfected MCF-10F cells were immunoprecipitated using 5 μg/ml of either normal rabbit IgG as a negative control (lane 1) or RelA-specific antibody (lanes 3,4). The resulting antibody-protein A-Sepharose eluates were subjected to immunoblot analysis with T7 epitope-specific antibody, as described above. Total cell lysate (30 μg protein) was analyzed as a positive control (lane 2). The 97-100 kDa T7-AhR product is indicated.

expression plasmid increased the transcriptional activity of the p1.6 Bgl promoter ~20-fold. These findings are consistent with the potent role of RelA in transactivation of the c-*myc* promoter in NIH 3T3 cells (31), and suggest an important functional role for the observed higher levels of RelA subunit expression in Hs578T cells in regulation of this oncogene (53). When the level of RelA expression plasmid was lowered 8-fold to 0.125 μg, a 2.4 +/- 0.5 fold increase in transactivation of the murine c-*myc* promoter was observed (Fig. 6A). This amount (0.125 μg) was selected for future transfections. Minimal transcriptional activity was observed when MCF-10F cells were transfected with p1.6 Bgl alone (Fig. 6A). This is consistent with the observation that c-*myc* gene transcription is minimal in cells at confluence. Transfection of 2 or 4 μg of AhR expression plasmid alone had no detectable effect on the transcriptional activity of the p1.6 Bgl promoter. However, when 0.125 μg *pEVRF-p65* were co-transfected with 2 or 4 μg of *pcDNA3-AhR* expression plasmid, a 6.2 +/- 0.3 and 8 +/- 0.2 fold induction of c-*myc* promoter activity, respectively, was seen. Thus, AhR is able to synergize with RelA to significantly increase c-*myc* promoter activity in MCF-10F cells.

We next asked whether the increase in transactivation of the c-*myc* promoter was mediated by the NF-κB elements located upstream of the promoter and/or within exon 1 (URE and IRE, respectively). A transfection experiment similar to that described above was performed using the p1.6 Bgl double mutant (p1.6 Bgl dbl mut) reporter plasmid in which the URE and IRE NF-κB sites have been mutated so that the promoter can no longer be transcriptionally activated by NF-κB. In the absence of exogenous RelA or AhR, the p1.6 bgl double mutant displayed about one half of the activity of the wild type p1.6 Bgl reporter (Fig. 6A). This modest decrease in activity of the mutant *vs* wild type p1.6 Bgl reporter construct is consistent with the low levels of RelA/p50 complexes present in the MCF-10F cells (56). Ectopically expressed RelA in *pEVRF-p65* transfected cells was unable to transactivate the mutant construct, consistent with our previous findings (31). Furthermore, co-transfection with 4 μg *pcDNA-3-AhR* and 0.125 μg *pEVRF-p65* did not significantly affect the activity of the mutated c-*myc* promoter (Fig. 6A). Taken together these findings indicate that RelA and AhR function synergistically to transactivate the c-*myc* promoter via binding at the URE and/or IRE NF-κB elements.

RelA and AhR activate the c-myc promoter in Hs578T cells-- We next asked whether RelA and AhR can activate the c-*myc* promoter in a human malignant breast cancer cell line by performing similar co-transfection analyses with Hs578T cells. In these cells the basal activity of the p1.6 Bgl

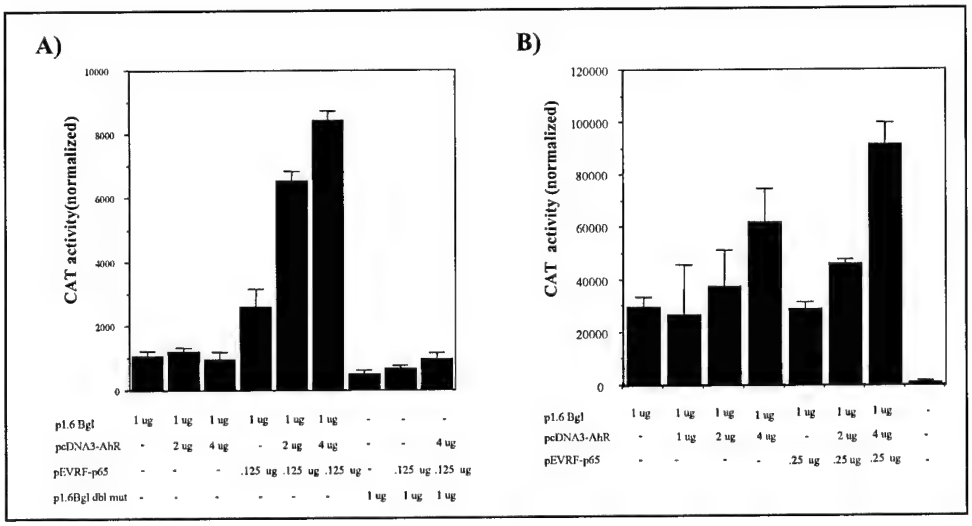

Fig. 6. **RelA and AhR cooperate to transactivate the wildtype p1.6Bgl, but not the p1.6Bgl dbl mut, c-*myc* promoter construct.** A) Confluent MCF-10F cells (~ 200,000 cells in 35 mm^2 dishes) were transiently transfected, in duplicate, with either 1 μg p1.6 Bgl or p1.6 Bgl dbl mut, and 0, 2, or 4 μg *pcDNA3-AhR* (murine AhR) expression vector in the absence or presence of 0.125 μg *pEVRF-p65* (RelA expression) plasmid using 7 μl FUGENE reagent. In each transfection, 1 μg of TK-luciferase plasmid was added as an internal control for normalization of transfection efficiency. Total DNA transfected was maintained at 6 μg by addition *pcDNA3* plasmid (parent vector for *pcDNA3-AhR*). Transfected cells were harvested after 24 h in reporter lysis buffer, and analyzed for CAT and luciferase activity. CAT activities are presented normalized for transfection efficiency, using the luciferase activity. B) Confluent Hs578T breast cancer cells were transiently transfected, in duplicate, with 1 μg of p1.6 Bgl plus 0, 1, 2, or 4 μg of *pcDNA3-AhR*, in the absence or presence of 0.25 μg of *pEVRF-p65* plasmid using 5 μl of FUGENE reagent. In each transfection, 0.5 μg of TK-luciferase plasmid was added and total DNA was maintained at 6 μg by addition of the appropriate amounts of *pcDNA3* plasmid. After 24 h, cells were harvested and analyzed for CAT and luciferase activities and protein levels. Values were normalized to protein levels because the TK-luciferase activity was not appreciable in these cells at confluence.

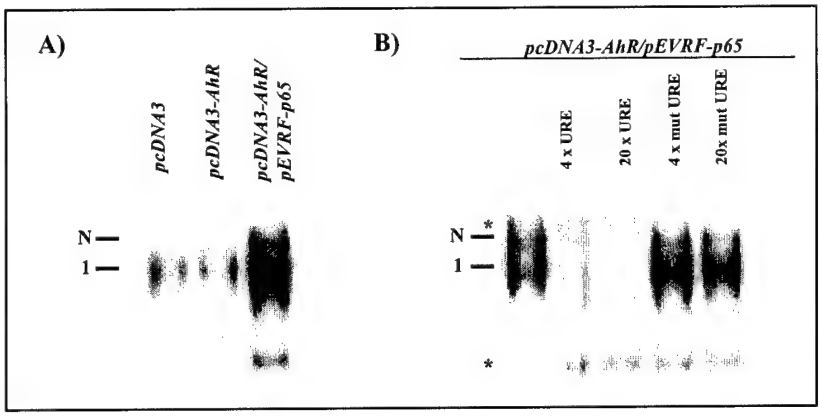

Fig. 7. **Expression of RelA and AhR yields a novel URE NF-κB element binding complex.** A) Co-transfection with AhR and RelA expression vectors leads to formation of a novel complex. Confluent plates (100 mm^2 dishes) of Hs578T cells were transfected with either 52 μg *pcDNA3* empty vector, or 50 μg *pcDNA3-AhR* in the absence or presence of 2 μg *pEVRF-p65* expression plasmid using 70 μl FUGENE reagent. After 24 h, nuclear proteins were isolated, and subjected to EMSA. N indicates position of a new complex; 1, indicates position of previously observed major complex. B) Competition EMSA confirms the specificity of the major bands. Nuclear extracts of Hs578T cells co-transfected with *pcDNA3-AhR* and *pEVRF-p65* were pre-incubated with either 4- or 20-fold molar excess unlabelled wildtype (URE) or mutant (mt URE) URE prior to the 30 min incubation reaction with the radiolabelled URE. Two nonspecific bands were identified and marked with an asterix (*).

promoter was notably higher than observed in transfected MCF-10F cells (Fig. 6B). This result likely reflects the higher transfection efficiency of Hs578T cells (20-30% *vs* 5% for MCF-10F cells), and potentially the higher endogenous levels of nuclear NF-κB/Rel proteins in these malignant cells (53). Interestingly, the activity of the p1.6 Bgl reporter plasmid increased in a dose-dependent fashion with transfection of increasing levels of AhR expression plasmid alone. Following transfection with 4 μg of *pc-DNA3-AhR*, CAT activity was 2.1 +/- 0.4 fold higher than basal levels. This result may be due to the effect of relatively high levels of constitutively active endogenous RelA protein present in these cells (see below). When a suboptimal dose (0.25 μg) of *pEVRF-p65* plasmid alone was added, no apparent change in p1.6 Bgl activity was seen. However, co-transfection of both the RelA and AhR expression plasmids resulted in induction of a higher level of c-*myc* promoter activity than was seen following transfection of either plasmid alone (Fig. 6B). Specifically, a 3.1 +/-0.3 fold induction of the c-*myc* promoter activity was observed following co-transfection with 4 μg *pc-DNA3-AhR* and 0.25 μg *pEVRF-p65* expression plasmids. The fact that the cooperative effects seen following AhR and RelA plasmid co-transfections in Hs578T cells were not as great as those seen in co-transfected MCF-10F cells may have been due to the higher level of background activity in the former cells, as well as the modest induction of reporter activity following transfection with 4 μg AhR expression plasmid alone in Hs578T cells.

AhR/RelA complexes bind to the URE NF-κB element-- To determine whether the AhR and RelA proteins are able to associate with the NF-κB binding elements in the c-*myc* gene, EMSA studies were performed using an oligonucleotide containing the NF-κB upstream regulatory element (URE) as probe. To enhance AhR and RelA expression, nuclear extracts from transfected Hs578T cells were used. (Hs578T cells were selected rather than MCF-10F cells because of the better transfection efficiency obtained with this line.) Confluent cultures of Hs578T cells were transfected using FUGENE with *pcDNA3-AhR* vector DNA in the absence or presence of *pEVRF-p65* RelA expression plasmid. As an additional control, cells were transfected with empty parental *pcDNA3* vector alone. Nuclear extracts, prepared 24 h post-transfection, were then used in EMSA. Since an

AhR/p65RelA complex might not bind DNA with the same affinity as typical NF-κB complexes, a lower dI:dC concentration was used to reduce the likelihood of competing away a specific binding complex. In control cells transfected with only parental *pcDNA3*, a major band migrating with the mobility of classic NF-κB was detected (labeled as band 1 in Fig. 7A). No change in the binding pattern was seen upon transfection with *pcDNA3-AhR*. When nuclear extracts from cells co-transfected with *pEVRF-p65* and *pcDNA3-AhR* expression plasmids were used, both the putative classic NF-κB band and a novel upper band (labeled "N") was seen (Fig. 7A). Equal loading of the lanes was confirmed in EMSA for an Oct-1 probe (data not shown). Addition of 4-fold or 20-fold molar excess wild type URE oligonucleotide successfully competed away complexes represented in both bands, whereas addition of similar amounts of mutant URE oligonucleotide, having the same two G to C conversions as in the p1.6 Bgl dbl mut construct (17,31), failed to compete (Fig. 7B).

To determine the subunit identities in the two specific binding complexes, supershift EMSA was performed using antibodies against either RelA or AhR. Addition of the AhR antibody specifically ablated band N without significantly changing the migration pattern of band 1 (Fig. 8A). In contrast, the AhR antibody had no effect on binding of nuclear proteins to an Oct-1 sequence (data not shown). Addition of the #1226 RelA-specific antibody (kindly provided by N. Rice, NCI) reduced formation of the complexes in both band 1 and band N (Fig. 8A). This p65 antibody also produced a faster migrating nonspecific band indicated by a double asterisk (Figure 8A), which was seen with the antibody and probe alone (data not shown). Thus, a second RelA-specific antibody (sc-372X) was used, which also clearly ablated formation of both band 1 and band N without manifestation of a faster migrating non-specific band (Fig. 8B). Addition of an antibody against the p50 subunit reduced band 1 and ablated a minor band below (Fig. 8B). An equivalent amount of a rabbit polyclonal antibody against an irrelevant protein (YY1, sc-281-X) failed to alter binding to the URE (data not shown). Thus, band 1 contains RelA and p50 proteins, and most likely represents binding of classical NF-κB heterodimers (RelA/p50). Based on its migration, the minor lower band likely consists of p50 homodimers. Band N contains both RelA and AhR proteins.

*AhR and RelA induces the endogenous c-*myc *gene--* To verify that the affects of AhR and RelA can be seen on chromosomal c-*myc* genes, co-transfection analysis was performed. Cultures of MCF-10F cells at 70% confluence were transfected with *pEVRF-p65* or *T7-pcDNA3-AhR* vector DNA alone or in combination. Whole cell extracts were prepared and subjected to immunoblot analysis for c-Myc protein (Fig. 8). While increases in the level of c-Myc protein were seen upon transfection of MCF-10F cells with either vector alone, a much greater induction was seen upon transfection of the combination of *pEVRF-p65* and *T7-pcDNA3-AhR* vector DNAs. The blot was also analyzed for β-actin protein, which confirmed equal loading. Using densitometry of this and a duplicate experiment, an increase in c-Myc level of 3.1+/-0.00-fold and 2.75+/-0.05-fold, respectively upon expression of RelA or AhR alone compared to control vector DNA was measured. An increase in c-Myc expression of 9.5+/-3.2-fold was observed upon co-transfection of both *pEVRF-p65* and *T7-pcDNA3-AhR* vector DNAs into MCF-10F cells. Presumably, the failure of AhR alone to induce the c-*myc* promoter construct above likely reflects deletion of the putative AhR binding elements, which are farther upstream. These results confirm the ability of RelA and AhR to cooperate in activation of the c-*myc* gene.

CONCLUSIONS AND FUTURE DIRECTIONS: In summary, our results show a physical and functional association of the AhR and the RelA subunit of NF-κB in transactivation of the c-*myc* gene in breast epithelial cells. Specifically, AhR and RelA were physically associated in malignant

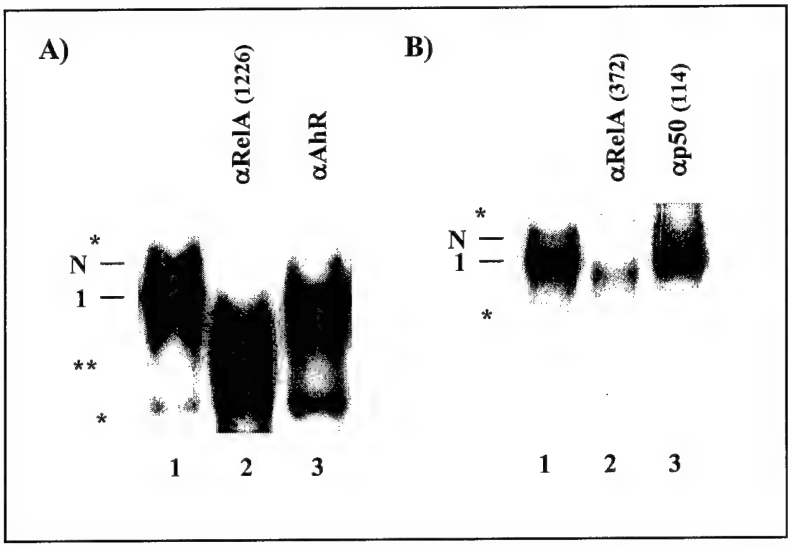

Fig. 8. **Novel NF-κB Binding Complex Contains AhR and RelA Protein.** Nuclear extracts from the AhR and RelA expression vector co-transfected cells, prepared as described above in Fig. 3, were incubated with the URE probe. Following a 30 min binding reaction, antibodies were added where indicated, the reactions incubated for an additional 1 h, and subjected to EMSA. Results of two separate analyses are shown. A) lane 1, no antibody; lane 2, 1 μl RelA-specific antibody (#1226, kindly provided by N. Rice), lane 3, 1 μl AhR-specific antibody (BioMol #SA-210); Two nonspecific bands were identified and marked with an asterix (*). A faster migrating, nonspecific band, that appears upon addition of antibody #1226 with the probe alone, is indicated by a double asterisk (**). B) lane 1, no antibody; 1 μl RelA-specific antibody (sc-372X); lane 3, 1 μl p50-specific antibody (sc-114). Specific binding complexes are indicated as band 1 and band N, as above.

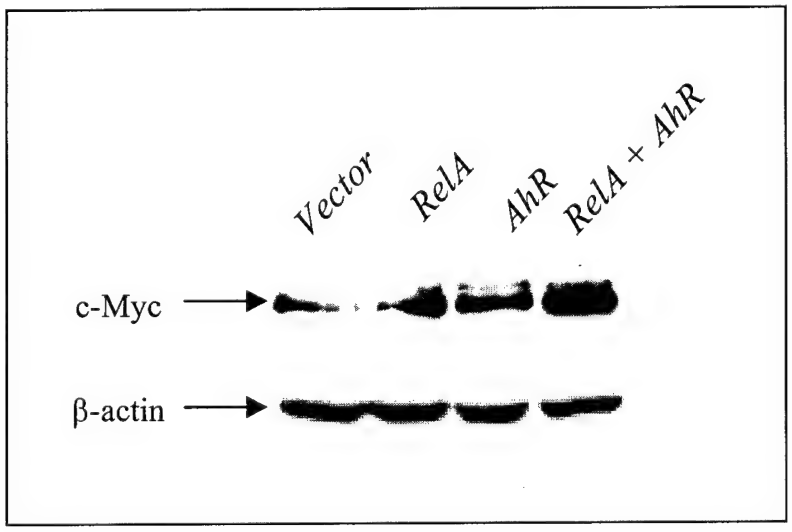

Fig. 9. **RelA and AhR cooperate to induce the endogenous c-*myc* gene in MCF-10F cells.** Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4 μg *pEVRF-p65* or 20 μg *T7-pcDNA3-AhR* DNA alone or in combination with 30 μl FUGENE transfection reagent. Total transfected DNA was maintained at 24 μg by addition of *pcDNA3* plasmid. Alternatively, cells were transfected with *pcDNA3* plasmid DNA alone (Vector). After 48 h, cells were harvested and samples of whole cell extracts (40 μg) subjected to immunoblot analysis for c-Myc (786-4) and β-actin (AC-15) proteins.

Hs578T breast cancer and non-malignant MCF-10F HMECs. AhR and RelA induced expression of the endogenous c-*myc* gene in MCF-10F cells. Using transfection analysis, AhR and RelA cooperated to transactivate the c-*myc* promoter in these two lines. As judged by transfection and mobility shift analyses, the RelA and AhR proteins formed a novel complex that binds to the wild type but not mutant NF-κB element of the c-*myc* gene. We postulate it is this complex, binding via the NF-κB element, that transactivates the c-*myc* promoter. Co-transfection of vectors that express AhR and RelA proteins with a wildtype *c-myc* promoter-reporter construct, but not with a promoter construct mutated in the NF-κB binding URE and IRE sites, led to increased levels of c-*myc* promoter transactivation. In contrast, cooperation between AhR and RelB or c-Rel subunits of NF-κB/Rel in transactivation of the c-*myc* gene was not observed in similar transfection analysis (data not shown). Furthermore, the novel transcription factor complex did not appear to contain the p50 subunit. Consistent with these findings, RelA but not p50 was found to specifically interact with the AhR in murine hepatoma cells (56). Thus, based on the relative mobility in EMSA, and the identified presence of both AhR and RelA in the novel complex, our results suggest that the AhR and RelA bind the URE as a heterotypic dimer composed of one subunit of each protein. Additional new studies are proposed to identify the domains of the RelA and AhR proteins mediating this interaction and transactivation.

Specific Aim 3: Monitor the effects of antioxidants known to inhibit NF-κB/Rel expression on cellular proliferation of transformed mammary cell lines.
a) Studies demonstrating the inhibitory effects of treatment with the antioxidant pentoxifylline (4) on growth of MCF-7 and 578T cells have been completed, as reported last year.
b) Green tea extracts have been found to be rich in antioxidants. Thus, we have analyzed the effects of two active components of green tea extacts, green tea polyphenols (GTP) and (-)-epigallocatechin-3-gallate (EGCG).
Growth curves-- To determine whether treatment with green tea extracts decreases the growth of Hs578T cells, which are estrogen receptor negative (ER-). Cells were treated with concentrations of green tea polyphenols or EGCG ranging from 0-160 ng/ul. Cell growth was assessed by cell numbers using a hemacytometer and by MTS assay, with essentially equivalent findings. Studies performed over a 72 hours treatment period showed that GTP slowed the growth of the Hs578T breast cancer cells. This effect was exhibited in a dose-dependent fashion, with 80 ng/ul GTP slowing growth, while higher doses (160 ng/ul) caused cell numbers to decrease (Fig. 10).

We next turned our attention to EGCG, the most potent polyphenolic component of green tea. Similar MTS analysis performed on Hs578T cells using EGCG, revealed that it too slowed the growth of the cells in a dose-dependent fashion (Fig. 11). At 40-80 ng/ul EGCG, cell growth slowed, while at higher dosages (160 ng/ul) a decline in cell numbers was observed.
Cell viability-- To determine whether the Hs578T cells were undergoing cell death or growth arrest, trypan blue exclusion analyses were performed. To ensure that dead, floating cells were not lost during the preparation, all media were collected and added back to the final cell volume. At dosages of 80 ng/ul, only a low percentage of cells took up the trypan blue dye, indicating that they were viable. In contrast, at 160 ng/ul of EGCG a significant level of death was detected (Fig. 12). A TUNEL assay implicated apoptosis in this cell death (data not shown). Therefore, EGCG is capable of killing cancer cells *in vitro* at a dose higher than those needed to arrest growth.

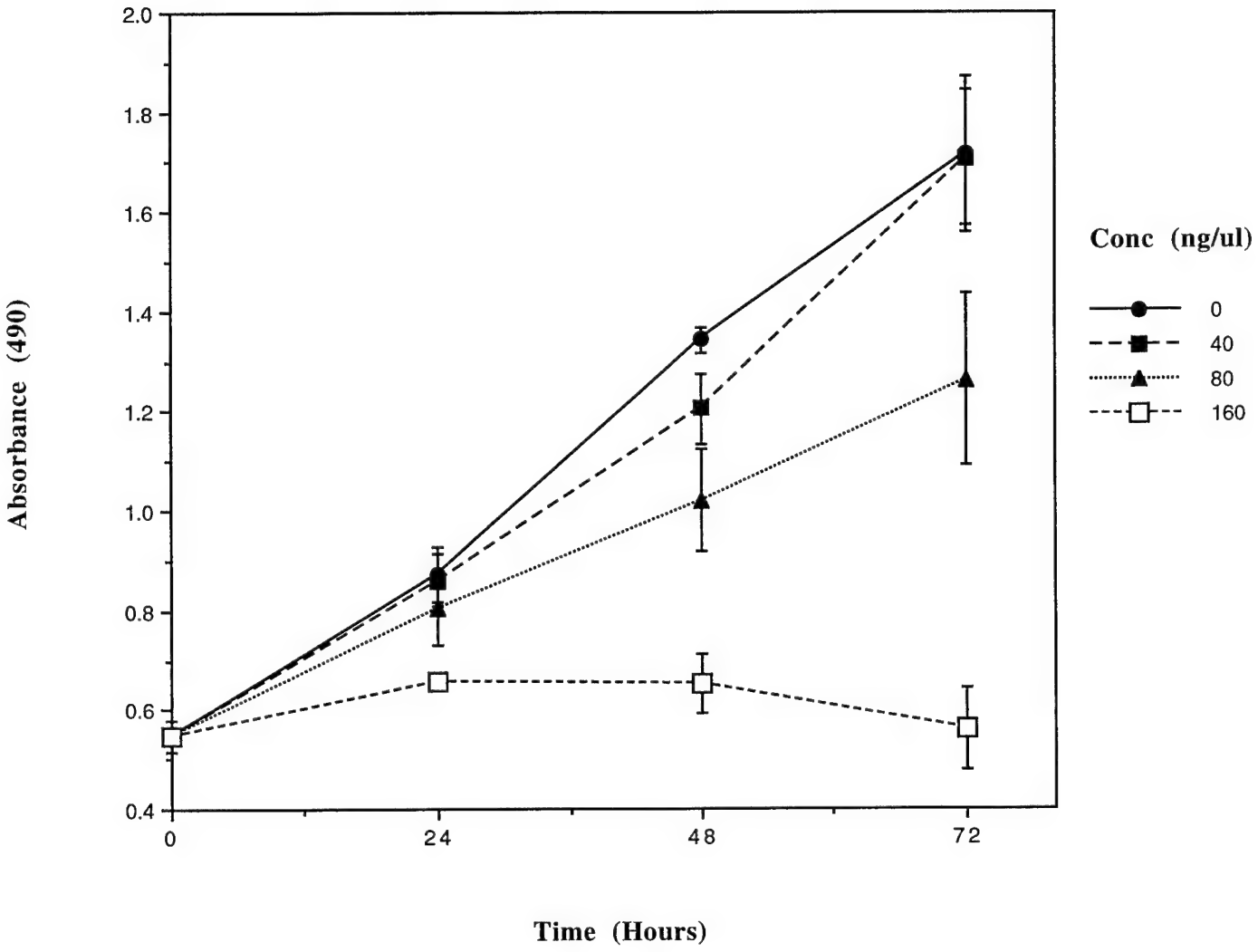

Fig. 10. **GTP decreases Hs578T breast cancer cell proliferation.** Hs578T cells were plated, in triplicate, at a density of 2,600 cells per cm^2. After overnight incubation, GTP was added at a final concentration of 0, 40, 80, or 160 ng/ul. An MTS viability assay performed after 24, 48 or 72 hours. The quantity of formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to the number of living cells in the culture.

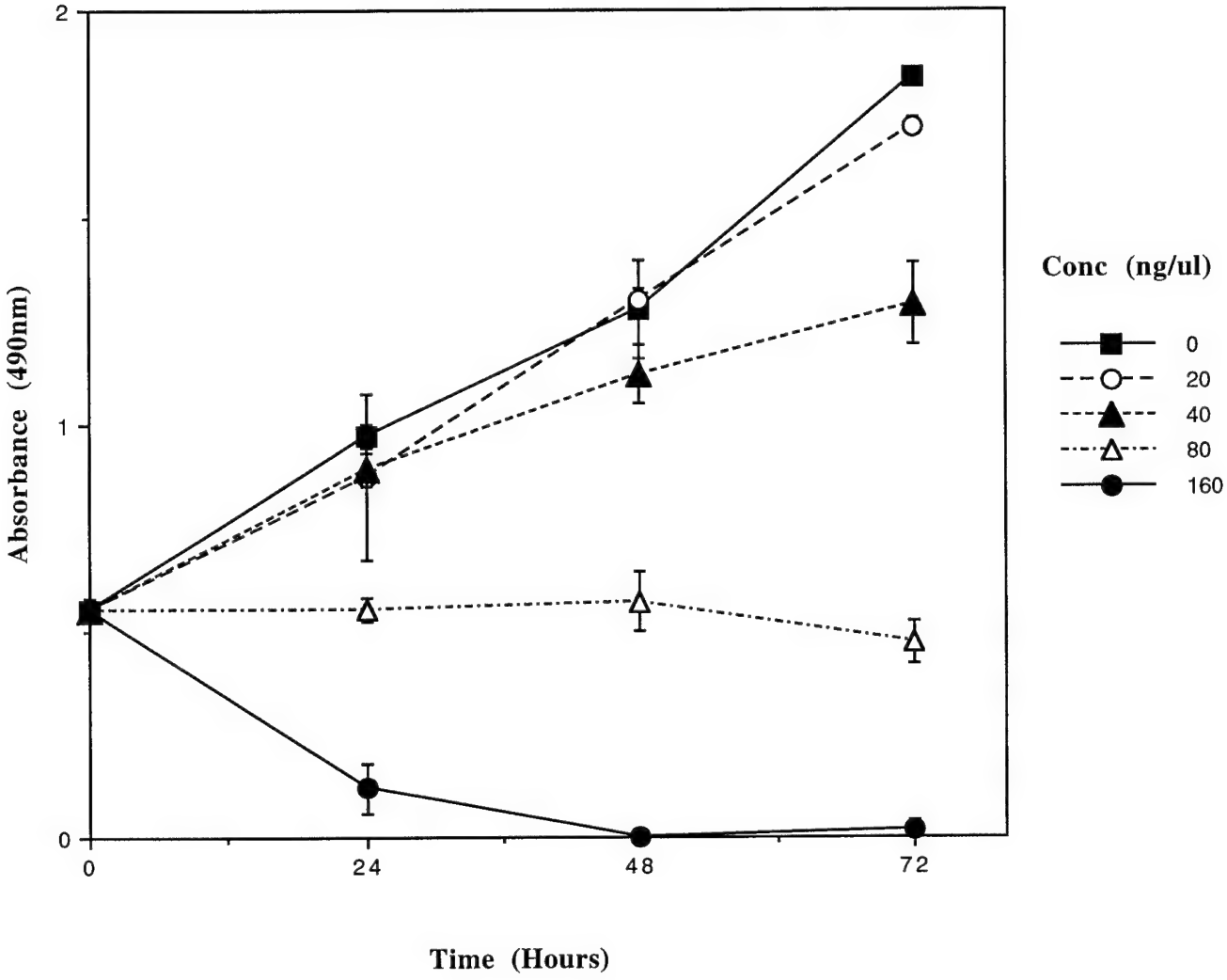

Fig. 11. **EGCG decreases Hs578T breast cancer cell proliferation.** Hs578T cells were plated, in triplicate, at a density of 2,600 cells per cm^2. After overnight incubation, EGCG was added at a final concentration of 0, 20, 40, 80, or 160 ng/ul. An MTS viability assay performed after 24, 48 or 72 hours.

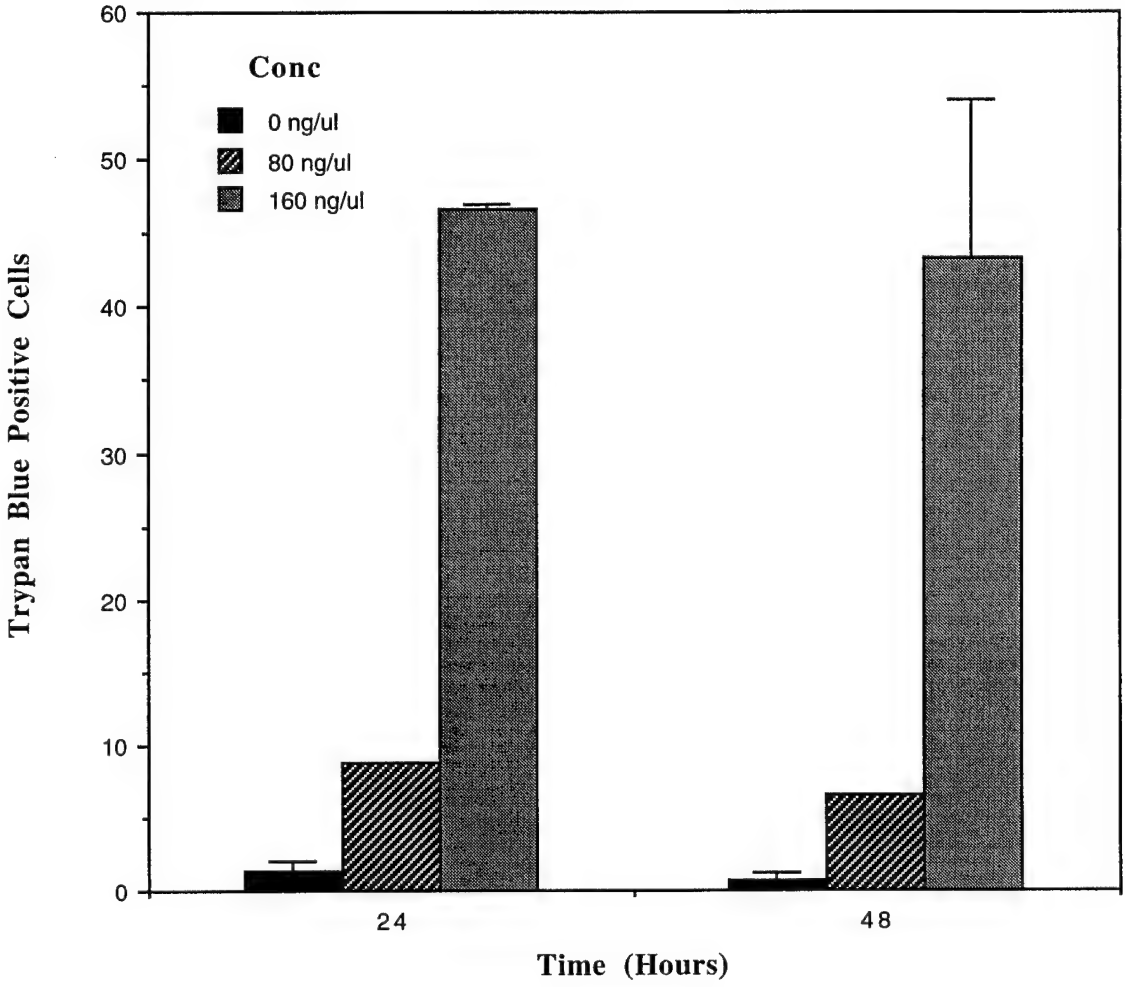

Fig. 12. **Dose-dependent effects of EGCG on Hs578T cell viability.** Hs578T cells were plated, in triplicate, at a concentration of 2,600 cells per cm^2. After overnight incubation, EGCG was added at a final concentration of 0, 80 or 160 ng/ul. The number of trypan blue positive cells were determined as a measure of lose of cell viability.

Taken together, these findings indicate that EGCG slows growth at 40 ng/ul, while at 80 ng/ul, it causes growth arrest and at 160 ng/ul apoptosis of Hs578T breast cancer cells.

Studies in other breast cancer cell lines-- We next sought to determine whether the effects of EGCG could be extended to other estrogen receptor negative (ER-) breast cancer cell lines. The medical community is searching for adjuvants that may be effective against ER- breast tumors, as these tumors are not responsive to anti-estrogenic treatments such as tamoxifen, and generally indicate a poorer prognosis. The effects of EGCG on MDA-MB-231 and D3-1 breast cancer cell proliferation was assessed using an MTS assay. The MDA-MB-231 cell line is a human cancer cell line derived from a patient with poorly differentiated adenocarcinoma of the breast. D3-1 is a carcinogen-transformed clonal cell line derived consequent to treatment of the MCF-10F cell line with 7,12-dimethylbenz(*a*)anthracene (DMBA). MDA-MB-231 cells displayed an EGCG dose-response curve similar to that of Hs578T cells, indicating that these cells are sensitive to EGCG growth retardation effects (Fig. 13). The D3-1 cell line appeared more sensitive to the effects of EGCG, responding to doses of 40 ng/ul EGCG (Fig. 14), and this was confirmed using trypan blue sensitivity assays (Fig. 15).

CONCLUSIONS: In summary, two components of great tea extracts, GTP and EGCG, which have been shown to have antioxidant properties, inhibit growth of three separate ER- breast cancer cell lines. These studies suggest drinking green tea may have a potential protective effect against breast cancer.

Specific Aim 4: Use transgenic mice to test the contribution of constitutive NF-κB/Rel subunit expression in the development of breast neoplasias.

a) During the previous period, we completed the preparation of an MMTV-c-Rel construct and the microinjection of mice. The mouse mammary tumor virus long terminal repeat (MMTV-LTR) is a hormonally responsive regulatory element that can direct expression of genes to the mammary epithelium as well as to other epithelial tissues. It is activated by corticosteroids and progestins; thus exogenous genes expressed in transgenic mice using the MMTV-LTR as a promoter are upregulated in females with each cycle of pregnancy.

b) During the past year, we have isolated and characterized six founder mice. Southern blot analysis of tail DNA is shown below for four of the lines (Fig. 16). Based on the DNA analysis of the six lines we have estimated the copy number of the founders as follows: Fo 7: 3.7; Fo8: 6.2; Fo 14: 4.7; Fo 15: 4.5; Fo16: 8.8; Fo18: 2.6. We have confirmed overexpression of c-Rel protein in mammary gland nuclei by immunoblot analysis (data not shown), and are in the process of breeding these lines to test for tumor formation.

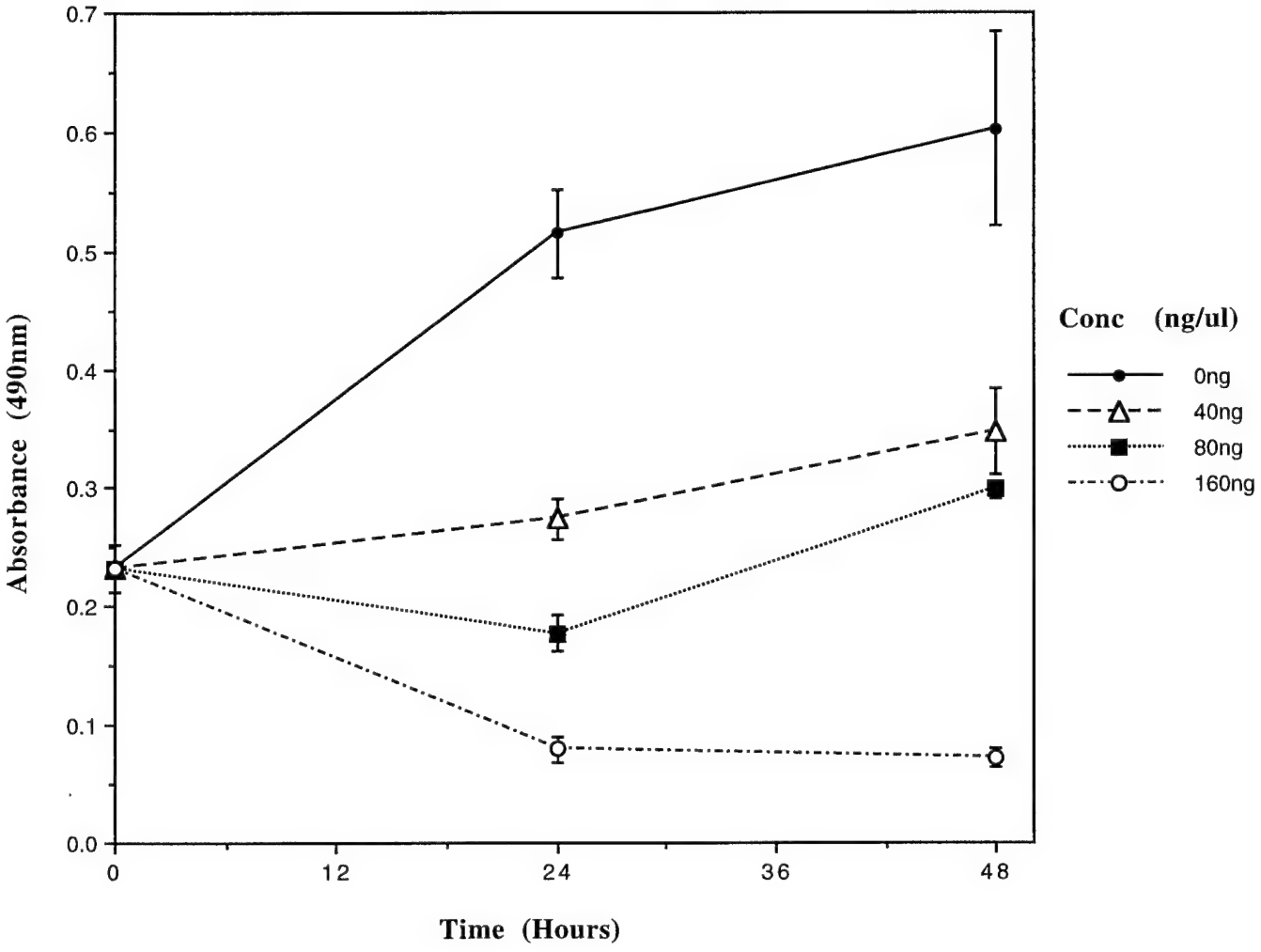

Fig. 13. **EGCG decreases MDA-MB-231 ER- breast cancer cell proliferation.** MDA-MB-231 cells were plated, in triplicate, at a concentration of 7,000 cells per cm^2. After overnight incubation, EGCG was added at a final concentration of 0, 20, 40, 80, or 160 ng/ul. An MTS viability assay performed, in duplicate, after 24, 48 or 72 hours.

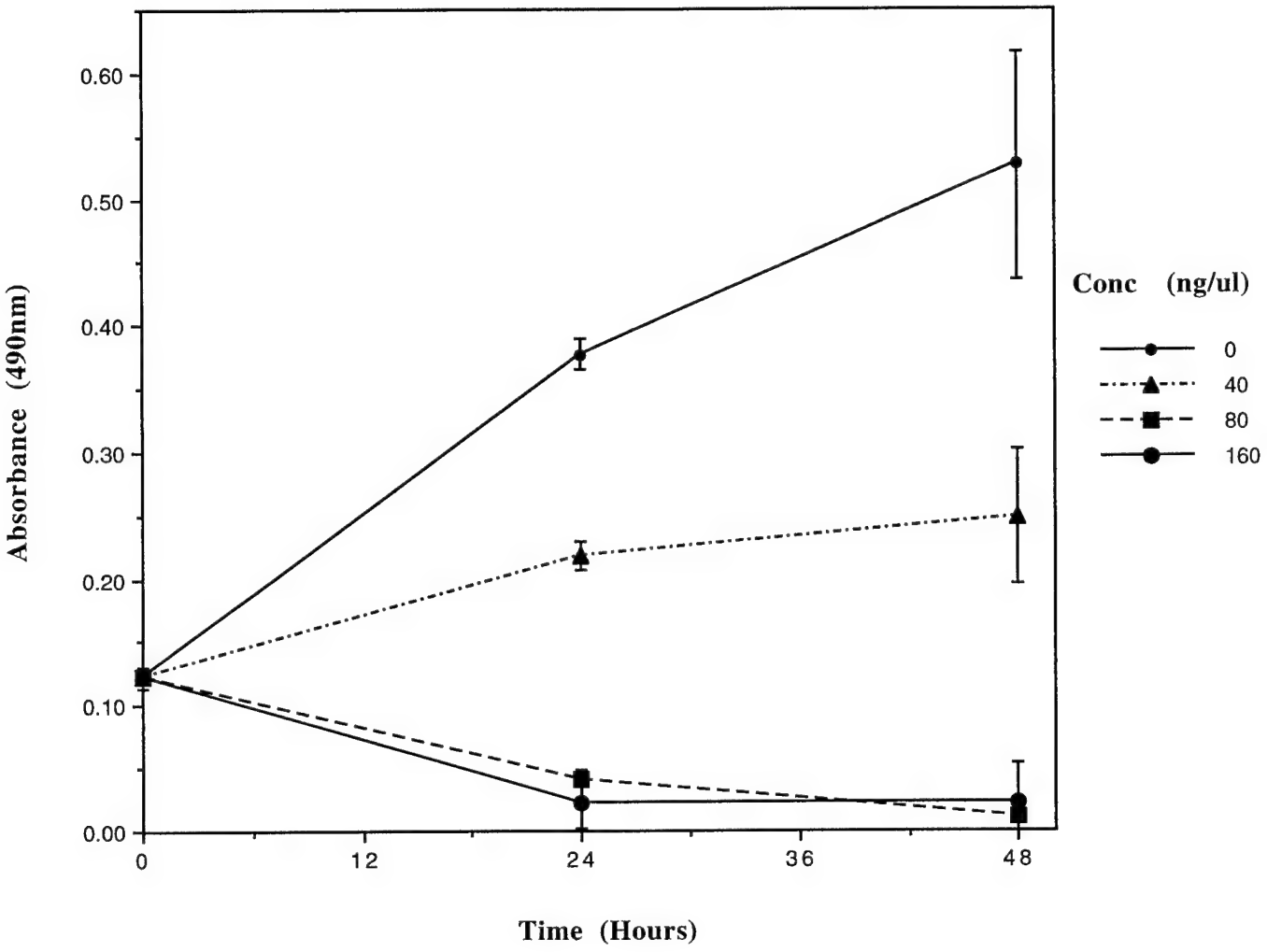

Fig. 14. **EGCG decreases D3-1 ER- breast cancer cell proliferation.** D3-1 cells were plated, in triplicate, at a concentration of 7,000 cells per cm^2. After overnight incubation, EGCG was added at a final concentration of 0, 20, 40, 80, or 160 ng/ul. An MTS viability assay performed, in duplicate, after 24, or 48 hours.

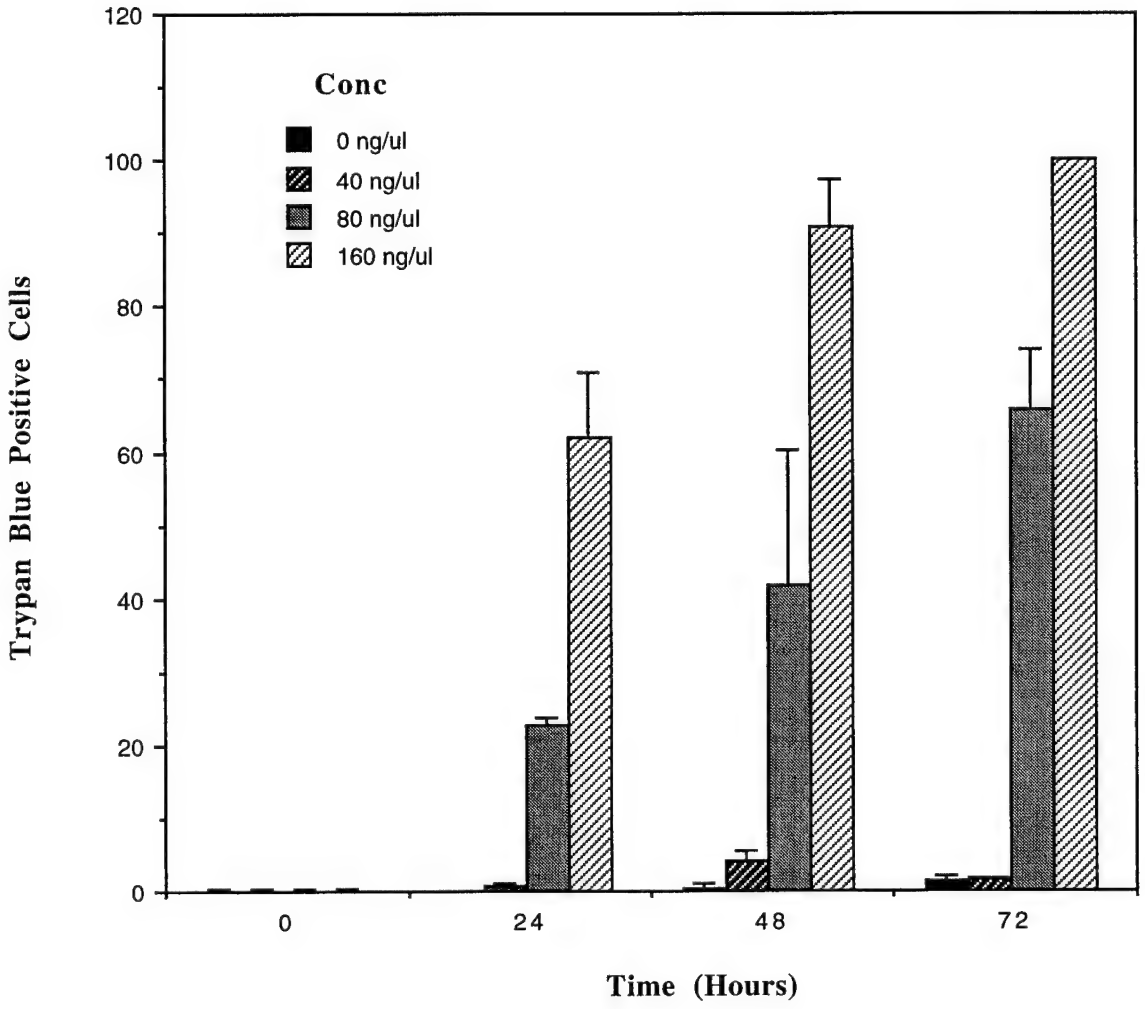

Fig. 15. **Dose-dependent effects of EGCG on D3-1 cell viability.** D3-1 cells were plated, in triplicate, at a concentration of 7,000 cells per cm^2. After overnight incubation, EGCG was added at a final concentration of 0, 40, 80, or 160 ng/ul. The number of trypan blue positive cells were determined as a measure of lose of cell viability.

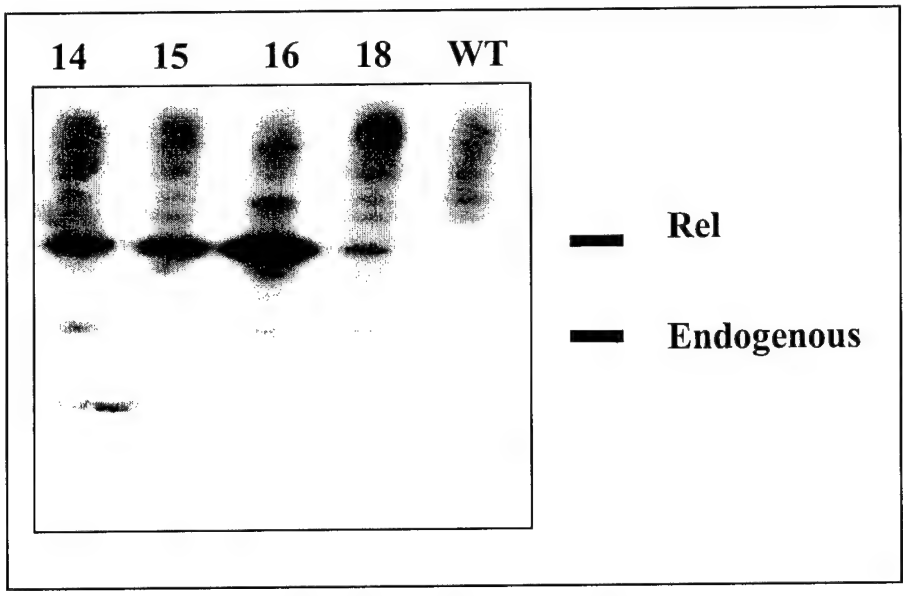

Fig. 16. **Southern blot analysis of DNA isolated from tails of MMTV-c-Rel founder mice.**

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated basal kinase activity of IKK and CK2 in breast cancer specimens.
- Showed a physical association of the aromatic hydrocarbon receptor/transcription factor (AhR) and the RelA subunit of NF-κB in breast epithelial cells.
- Demonstrated a functional interaction between the AhR and Rel A subunit in transactivation of the c-*myc* gene in breast epithelial cells.
- Showed the RelA and AhR proteins formed a novel complex that binds to the wild type but not mutant NF-κB element of the c-*myc* gene, indicating a novel signaling pathway.
- Demonstrated two components of great tea extracts, GTP and EGCG, which have been shown to have antioxidant properties, inhibit growth of three separate ER- breast cancer cell lines.
- Prepared MMTV-c-Rel transgenic mice for *in vivo* study of the role of NF-κB in the etiology of breast cancer.

REPORTABLE OUTCOMES

Manuscripts:

1. Sovak, M.A., M. Arsura, G. Zanieski, K.T. Kavanagh, and G.E. Sonenshein, The inhibitory effects of transforming growth factor-β1 on breast cancer cell proliferation are mediated through regulation of aberrant NF-κB/Rel expression. Cell Growth Diff. 10, 537-544 (1999).
2. Kim, D.W., M.A. Sovak, G. Zanieski, G. Nonet, R. Romieu-Mourez, A.W. Lau, L.J. Hafer, P. Yaswen, M. Stampfer, A.E. Rogers, J. Russo, and G.E. Sonenshein. Activation of NF-κB/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis (in press).
3. D.W. Kim, L. Gazourian, S.A. Quadri, R. Romieu-Mourez, D.H. Sherr, and G.E. Sonenshein. The Aryl Hydrocarbon Receptor/Transcription Factor (AhR) and the Rel A Nuclear Factor-κB subunit cooperate to transactivate the c-*myc* promoter. (manuscript submitted).

Poster:
Mammary Gland Gordon conference, June 6-11, 1999.

Mouse lines:
Prepared MMTV-c-Rel transgenic mice for *in vivo* study of the role of NF-κB in the etiology of breast cancer

Degrees obtained:
Dong W. Kim, Ph.D. degree (returned to medical school to complete MD degree).

Funding applied for based on work supported by this award:
"Role of NF-κB/Rel in the Pathogenesis of Breast Cancer", G.E. Sonenshein; NIH/NCI; RO1: 9/1/99-6/30/04: Specific Aims: 1) Determine the functional role of constitutive NF-κB/Rel activity in breast cancer cells, using transgenic mice expressing a NF-κB subunit and IκB-α; 2) Evaluate the kinase activity in tumor cell lines in culture; 3) Determine the functional role of the c-*myc* oncogene as a target of NF-κB/Rel activation in development of breast neoplasias, including signals mediating growth, apoptosis and neoplastic transformation of cells; 4) Determine whether aberrant activation of NF-κB/Rel occurs prior to neoplastic transformation in human breast disease using immunohistochemistry of primary premalignant atypical hyperplasia.

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The Inhibitory Effects of Transforming Growth Factor β1 on Breast Cancer Cell Proliferation Are Mediated through Regulation of Aberrant Nuclear Factor-κB/Rel Expression[1]

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Abstract

Nuclear factor (NF)-κB/Rel transcription factors normally exist in non-B cells, such as epithelial cells, in inactive forms sequestered in the cytoplasm with specific inhibitory proteins, termed IκBs. Recently, however, we discovered that breast cancer is typified by aberrant constitutive expression of NF-κB/Rel factors. Because these factors control genes that regulate cell proliferation, here we analyzed the potential role of NF-κB/Rel in the ability of transforming growth factor (TGF)-β1 to inhibit the growth of breast cancer cells. The decreased growth of Hs578T and MCF7 breast cancer cell lines on TGF-β1 treatment correlated with a drop in NF-κB/Rel binding. This decrease was due to the stabilization of the inhibitory protein IκB-α. Ectopic expression of c-Rel in Hs578T cells led to the maintenance of NF-κB/Rel binding and resistance to TGF-β1-mediated inhibition of proliferation. Similarly, expression of the p65 subunit ablated the inhibition of Hs578T cell growth mediated by TGF-β1. Thus, the inhibition of the aberrantly activated, constitutive NF-κB/Rel plays an important role in the arrest of the proliferation of breast cancer cells, which suggests that NF-κB/Rel may be a useful target in the treatment of breast cancer.

Introduction

TGF-β1[3] belongs to a family of polypeptides that plays a role in cellular proliferation, development, and extracellular matrix modeling. TGF-β1 has been demonstrated to have significant inhibitory effects on the growth of numerous cell types (1–3), including mammary epithelial cells. *In vitro* studies have revealed its inhibitory effects upon the proliferation of numerous primary human and established breast epithelial cell lines (4, 5). Evidence for a role of TGF-β1 in normal mammary gland development and proliferation was provided by studies in mice. Transgenic mice expressing TGF-β1 linked to the MMTV promoter displayed high levels of TGF-β1 in the mammary gland (6). These mice were demonstrated to have hypoplastic mammary duct development (6). In addition, Silberstein and Daniel (7) demonstrated that the temporary placement of slow-release TGF-β1 pellets in the mammary glands of virgin mice reversibly inhibited both mammary ductal growth and DNA synthesis.

The growth inhibitory actions of TGF-β1 have also been demonstrated in breast tumor cell lines (4, 8–10). This effect has been observed in both ER-positive cells (MCF7) and ER-negative cells (Hs578T; Ref. 8), although some studies have suggested that ER-negative cells, which are often found in more advanced breast cancers, are less susceptible to the effects of TGF-β1 (reviewed in Ref. 11). The effects of TGF-β1 on breast cancer have also been seen *in vivo*. For example, the MMTV-TGF-β1 transgenic mice displayed increased resistance to the breast carcinogenic effects of DMBA (6). Furthermore, when these mice were crossed with MMTV-TGF-α transgenic mice, which show increased incidence of both spontaneous and DMBA-induced breast tumors, the offspring displayed a decreased incidence of spontaneous breast tumors as well as a resistance to the tumorigenic effects of DMBA compared with the parental TGF-α transgenics (6). These studies suggest that TGF-β1 significantly inhibits the development of breast cancer.

There are several theories as to the mechanism of action of TGF-β1. Studies have linked its growth-inhibitory effects to the down-regulation of genes involved in cellular proliferation, such as those encoding cyclin-dependent kinases (12–14), the retinoblastoma susceptibility product (pRB) (15, 16), and the c-Myc oncoprotein (16, 17). Recent results from our laboratory have shown that the TGF-β1 treatment of immature B cells and hepatocytes involves a novel signaling mechanism exerted through down-regulation of the NF-κB/Rel family of transcription factors (18, 19). NF-κB/Rel is a family of dimeric transcription factors all of whose members

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[3] The abbreviations used are: TGF-β1, transforming growth factor β1; MMTV, mouse mammary tumor virus; ER, estrogen receptor; DMBA, 7,12-dimethylbenz(α)anthracene; NF, nuclear factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium inner salt; Oct-1, Octomer-1; ATCC, American Type Culture Collection; LP, leupeptin; buffer C, 420 mM KCl, 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 20% glycerol; PMSF, phenylmethylsulfonyl fluoride; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay.

contain a 300-amino-acid Rel homology domain (20, 21). In mammalian cells, subunit members include p50 or NF-κB1, p52 or NF-κB2, p65 or RelA, c-Rel, and RelB (20). NF-κB/Rel factors are involved in the control of genes implicated in the regulation of cellular proliferation, cell survival, adhesion, and immune and inflammatory responses (20, 21). The activity of NF-κB/Rel factors is controlled posttranslationally by their subcellular localization. In most cells, other than mature B lymphocytes, NF-κB/Rel proteins are sequestered as inactive forms in the cytoplasm by association with inhibitory proteins, termed IκB's, for which IκB-α represents the prototype (22–25). Activation involves IκB phosphorylation, which results in its ubiquitination and subsequent degradation, which then allows for the nuclear translocation of the NF-κB/Rel protein (25). Recently, we reported that NF-κB/Rel is aberrantly activated in human breast cancer and in rat mammary tumors induced by DMBA (26). Thus, here we have investigated the role of NF-κB/Rel in the TGF-β1-mediated inhibition of Hs578T and MCF-7 breast cancer cell proliferation. We report that the inhibition of cell growth on TGF-β1 treatment of these tumor cell lines correlates with a decrease in NF-κB/Rel activity due to increased stability of IκB-α protein. Furthermore, ectopic expression of c-Rel or p65 in Hs578T cells ablates the TGF-β1-mediated decrease in NF-κB/Rel activity and inhibition of growth. These findings provide evidence for a direct role of NF-κB/Rel in the TGF-β1-mediated decrease in the proliferation of breast cancer cells.

Results

TGF-β1 Decreases Breast Tumor Cell Line Proliferation. To confirm the effects of TGF-β1 treatment on the proliferation of Hs578T and MCF7 breast cancer cells, the effects of this cytokine on cell numbers were monitored. Concentrations of TGF-β1 ranging from 1 to 5 ng/ml have been reported to effectively inhibit breast cancer cell proliferation (4, 8, 27, 28). Exponentially growing Hs578T and MCF7 cells were, therefore, treated for 72 h with either TGF-β1 dissolved in 4% BSA carrier solution or carrier solution alone as control. Cell numbers were then determined. As can be seen in Fig. 1, TGF-β1 treatment for 3 days resulted in fewer MCF7 and Hs578T cells compared with controls. Similarly, percent labeled nuclei values were largely reduced in Hs578T or MCF7 cells that had been treated with TGF-β1 for 72 h compared with BSA-treated control cells (data not shown). The effects of TGF-β1 were likely due to the inhibition of cellular proliferation as opposed to the induction of cell death because a visual analysis of nuclear morphology did not reveal a significant number of apoptotic cells (data not shown). Thus, as seen previously (4, 5), TGF-β1 potently inhibits the growth of breast cancer cell lines.

TGF-β1 Decreases NF-κB/Rel Expression. TGF-β1 has been demonstrated to exert its inhibitory effects on proliferation through numerous mechanisms, including through the down-regulation of NF-κB/Rel activity (18–19). To determine whether TGF-β1 affects the aberrant NF-κB/Rel activity in breast cancer cells, an EMSA analysis was performed. Nuclear extracts were prepared from Hs578T and MCF7 cells incubated in the presence of 1 ng/ml TGF-β1 or carrier BSA

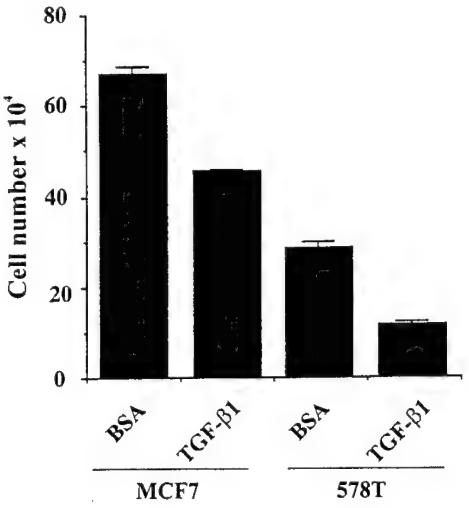


Fig. 1. TGF-β1 treatment decreases proliferation of breast tumor cell lines. MCF7 and Hs578T (578T) breast tumor cell lines were plated in duplicate and treated with 1 ng/ml TGF-β1 or carrier BSA solution as control. Cell numbers were determined after 3 days of treatment.

for 72 h. The upstream NF-κB element from the c-*myc* gene (URE) was used as probe (29). As shown in Fig. 2*A*, TGF-β1 treatment decreased the total levels of NF-κB/Rel binding activity in both cell lines. To determine the kinetics of the TGF-β1-mediated down-modulation of NF-κB/Rel activity, Hs578T cells were treated for 24 or 48 h with TGF-β1 and analyzed by EMSA. As shown in Fig. 2*B*, TGF-β1 treatment caused a significant decrease of NF-κB/Rel binding after 24 h, which was followed by an even more pronounced down-modulation at the 48-h time point. These effects were specific in that TGF-β1 treatment did not alter Oct-1 binding (Fig. 2*C*). Thus, TGF-β1 decreases both breast tumor cell proliferation and NF-κB/Rel binding activity.

TGF-β1 Increases the Half-Life of IκB-α Protein. In hepatocyte cell lines, the decrease in NF-κB activity in response to TGF-β1 treatment was mediated through an increase in IκB-α protein specifically; the levels of IκB-β, the other major IκB protein, were unaffected (19). Thus, the effects of TGF-β1 treatment of Hs578T cells on the rate of turnover of IκB proteins was assessed. Hs578T cells were incubated for 48 h in the presence of TGF-β1 or BSA carrier solution as control, and then treated with the protein synthesis inhibitor emetine for 1, 2, or 4 h. Cytoplasmic extracts were then subjected to immunoblot analysis for the two predominant IκB proteins, IκB-α and IκB-β (Fig. 3*A*). A significant decrease in IκB-α degradation was noted upon TGF-β1 treatment. Densitometry was performed on the resulting immunoblots, and the relative levels were plotted as a function of time in Fig. 3*B*. In the BSA-treated cells, IκB-α protein had a half-life of decay of approximately 2.75 h (Fig. 3*B*). TGF-β1-treatment increased the normal half-life beyond 4 h. In contrast, IκB-β protein had a longer half-life, and no change was detected over the 4-h time course (Fig. 3*A*). TGF-β1 treatment of MCF-7 cells similarly increased the half-life of IκB-α compared with BSA-carrier-treated control cells, *i.e.*, a 12-h half-life was determined after 48 h of

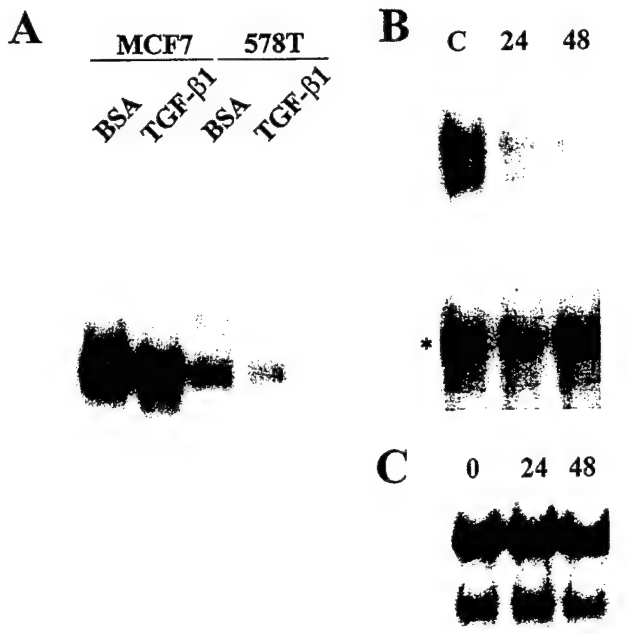


Fig. 2. TGF-β1 treatment decreases nuclear NF-κB/Rel binding activity in breast tumor cell lines. In *A*, NF-κB binding. MCF7 and Hs578T (*578T*) breast tumor cell lines were plated at 1.1×10^4 cells/P100 or 5.6×10^4 cells/P100, respectively, and treated for 3 days with 1 ng/ml TGF-β1 or carrier BSA solution as control. Nuclear extracts were made and subjected to EMSA using as probe the URE NF-κB oligonucleotide. *B*, NF-κB binding. Hs578T breast tumor cell lines were treated with carrier BSA for 48 h (*C*) or with 1 ng/ml TGF-β1 for 24 or 48 h, as indicated. Nuclear extracts were prepared and processed as in *A*. *, a nonspecific band that did not change with TGF-β1 treatment. *C*, Oct-1 binding. Hs578T breast tumor cell lines were treated with 1 ng/ml TGF-β1 for 0, 24, or 48 hrs. Nuclear extracts were prepared and subjected to EMSA for Oct-1 binding.

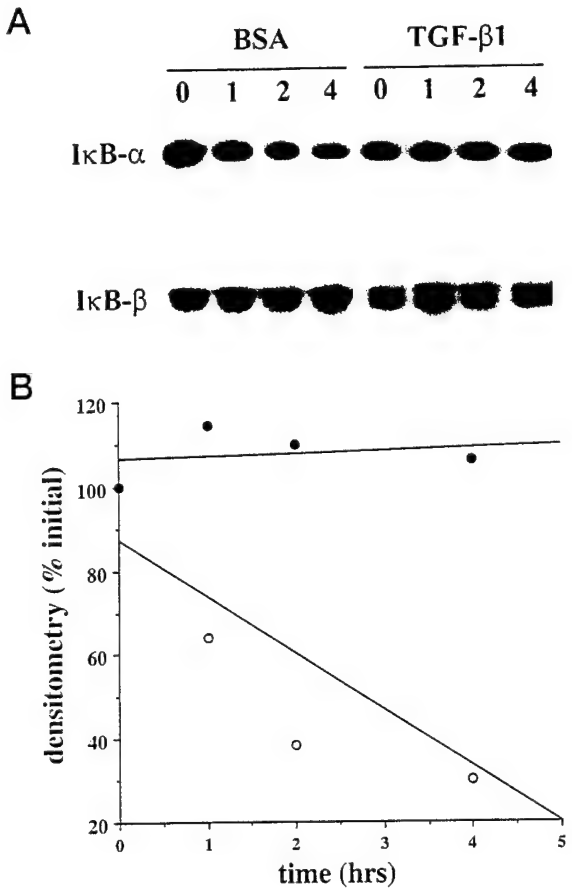
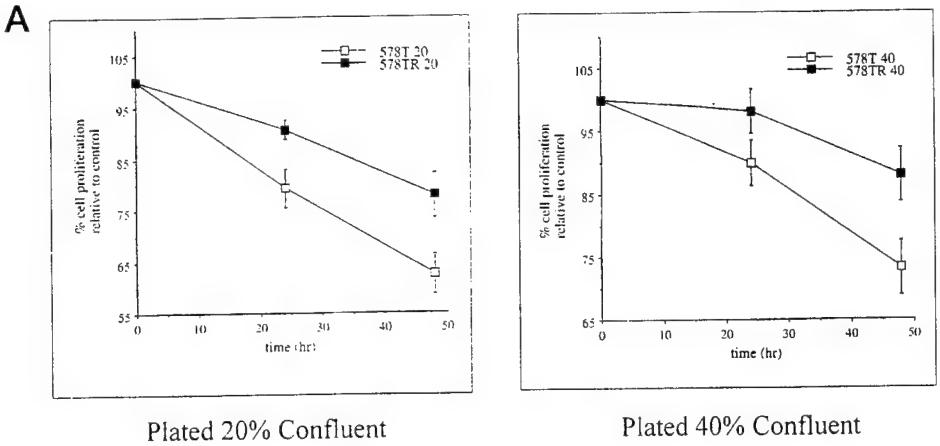


Fig. 3. TGF-β1 treatment increases the half-life of decay of IκB-α in Hs578T breast cancer cells. In *A*, cultures of Hs578T cells were plated at a density of 2×10^5 cells/P100 dish the day before treatment. Cells were treated with 5 ng/ml TGF-β1 or carrier solution for 48 h and then incubated in the presence of the protein synthesis inhibitor 20 μg/ml emetine for 0, 1, 2, or 4 h. Cytoplasmic extracts were prepared, and equal amounts (30 μg) were subjected to immunoblot analysis for IκB-α or IκB-β. In *B*, the autoradiograph for a representative experiment for IκB-α was quantified by densitometric analysis, and the data were presented as a function of the amount present at time zero, set at 100% for the control and TGF-β1-treated samples. ○, BSA control; ●, treated with TGF-β1.

TGF-β1 treatment compared with the normal 5.3 hrs in control cells. Thus, TGF-β1 treatment stabilizes IκB-α protein, lengthening the normal rate of decay.

Ectopic c-Rel Expression Ablates the Inhibitory Effects of TGF-β1 on Hs578T Cells. To test whether ectopic expression of a transactivating NF-κB/Rel subunit was sufficient to rescue breast cancer cells from TGF-β1-induced cell growth arrest, populations of Hs578T cells expressing ectopic c-Rel were prepared. To this purpose, the murine c-Rel expression vector pSV-SPORT-c-Rel, which encodes a full-length c-Rel protein, was chosen. Hs578T cells were then stably transfected with the pSV-SPORT-c-Rel vector and the neomycin resistance construct pSV_2neo DNA and selected for G418 resistance, as described in "Materials and Methods." Two individual clonal lines were then isolated from the mixed population of resistant cells by limiting dilution. Although studies with individual clones usually give more substantial effects, the use of mixed populations confirms that an observation is not specific to only a few individual cells within a population. The mixed population c-Rel-transfected Hs578T cells (Hs578TR) were first tested for the extent of cell growth inhibition after TGF-β1 treatment, as compared with the response of the parental Hs578T cell line, using an MTS conversion assay. Treatment of Hs578T cells, plated at either 20 or 40% confluence, with TGF-β1 for 24 or 48 h resulted in a significant decrease in cell proliferation (Fig. 4*A*). In contrast, TGF-β1 treatment had significantly more modest effects on the cell proliferation of Hs578TR cells (Fig. 4*A*). The Hs578TR cells were next characterized for the effects of TGF-β1 treatment on NF-κB/Rel binding activity (Fig. 4*B*). As expected, NF-κB/Rel binding was dramatically diminished after TGF-β1 treatment of the parental Hs578T cells for 24 and 48 h. The nuclear extracts from untreated Hs578TR cells displayed a higher level of NF-κB/Rel binding activity compared with those from the parental Hs578T cells (Fig. 4*B*). Furthermore, the nuclear extracts from Hs578TR cells retained significantly higher levels of NF-κB/Rel binding activity even after TGF-β1 treatment. These studies demonstrate that ectopic expression of a member of the NF-κB/Rel family prevents the drop in NF-κB/Rel and rescues cells from the growth inhibition mediated by TGF-β1.

The clones were next monitored specifically for c-Rel expression by immunoblotting. Cultures of individual clones of



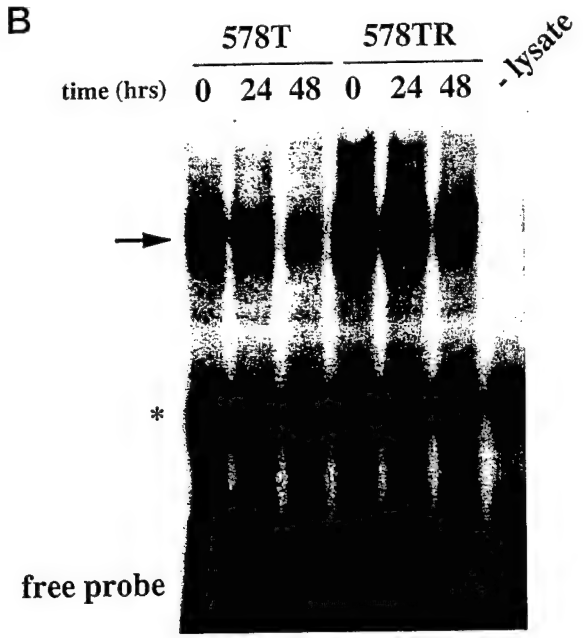


Fig. 4. Ectopic c-Rel expression in the Hs578TR cell population. Cultures of Hs578T cells in exponential growth were transfected with 22.5 μg of the murine c-Rel expression vector pSPORT-c-Rel and 2.5 μg of pSV_2neo DNA. G418-resistant stable transfectants were isolated. In *A*, parental Hs578T and Hs578TR cells were plated at 20 and 40% confluence, and treated with 2 ng/ml TGF-β1 or BSA as control for 24 and 48 h, respectively. The effects of TGF-β1 on growth were measured by MTS assay. Cell numbers for TGF-β1-treated cells are given as percent values relative to BSA-treated control cells. *B*, transfected Hs578TR (*578TR*) and parental Hs578T (*578T*) cells were plated at 40% confluence and treated with 2 ng/ml TGF-β1 for 24 and 48 h. Nuclear extracts were isolated and subjected to EMSA using the URE NF-κB oligonucleotide as probe. *, a nonspecific band that did not change with TGF-β1 treatment.

the Hs578TR and parental Hs578T cells were compared for expression of c-Rel (Fig. 5*A*). The parental Hs578T cells contained extremely low levels of c-Rel, in agreement with our previous report (26). All of the individual clones expressed c-Rel at levels significantly higher than the parental Hs578T cells. Several of the clones (Hs578TR-C1, -C2, and -C5) expressed high levels of c-Rel, whereas Hs578TR-C3 and -C4 expressed moderately increased levels compared with the parental line. Specificity of the c-Rel protein detected was confirmed by competition with the cognate peptide (Fig. 5*B*). The effects of TGF-β1 on growth were further explored using the two individual c-Rel expressing clones Hs578TR-C1 and -C2. The effects of TGF-β1 treatment were monitored in two ways, MTS assay and percent labeled nuclei. TGF-β1-mediated inhibition of cell proliferation was almost completely ablated in cultures plated at 20% confluence (Fig. 5*C*) or at 70% confluence (data not shown). Similarly, the TGF-β1 mediated inhibition of DNA synthesis was significantly ablated by expression of c-Rel (Fig. 5*D*). Thus, the maintenance of higher levels of NF-κB/Rel activity protects cells from TGF-β1-induced growth arrest.

Ectopic Expression of p65 Ablates the Growth Inhibitory Effects of TGF-β1. To determine whether expression of the p65 subunit would similarly alter the response of Hs578T cells to TGF-β1, these cells were transiently transfected using FUGENE (Roche Diagnostics Corp., Indianapolis, IN) reagent, which permits a much higher efficiency of transfection. Cells were transfected either with a human p65 pMT2T expression vector or with the parental pMT2T vector DNA. A GFP expression vector was added to estimate transfection efficiency, which was estimated at approximately 70% based on GFP staining. After a 48-h treatment with TGF-β1, cell proliferation was assessed by MTS assay, and the average of two experiments, carried out in triplicate, are shown in Fig. 6. The reduction of Hs578T cell proliferation normally seen on TGF-β1 treatment was greatly ablated on

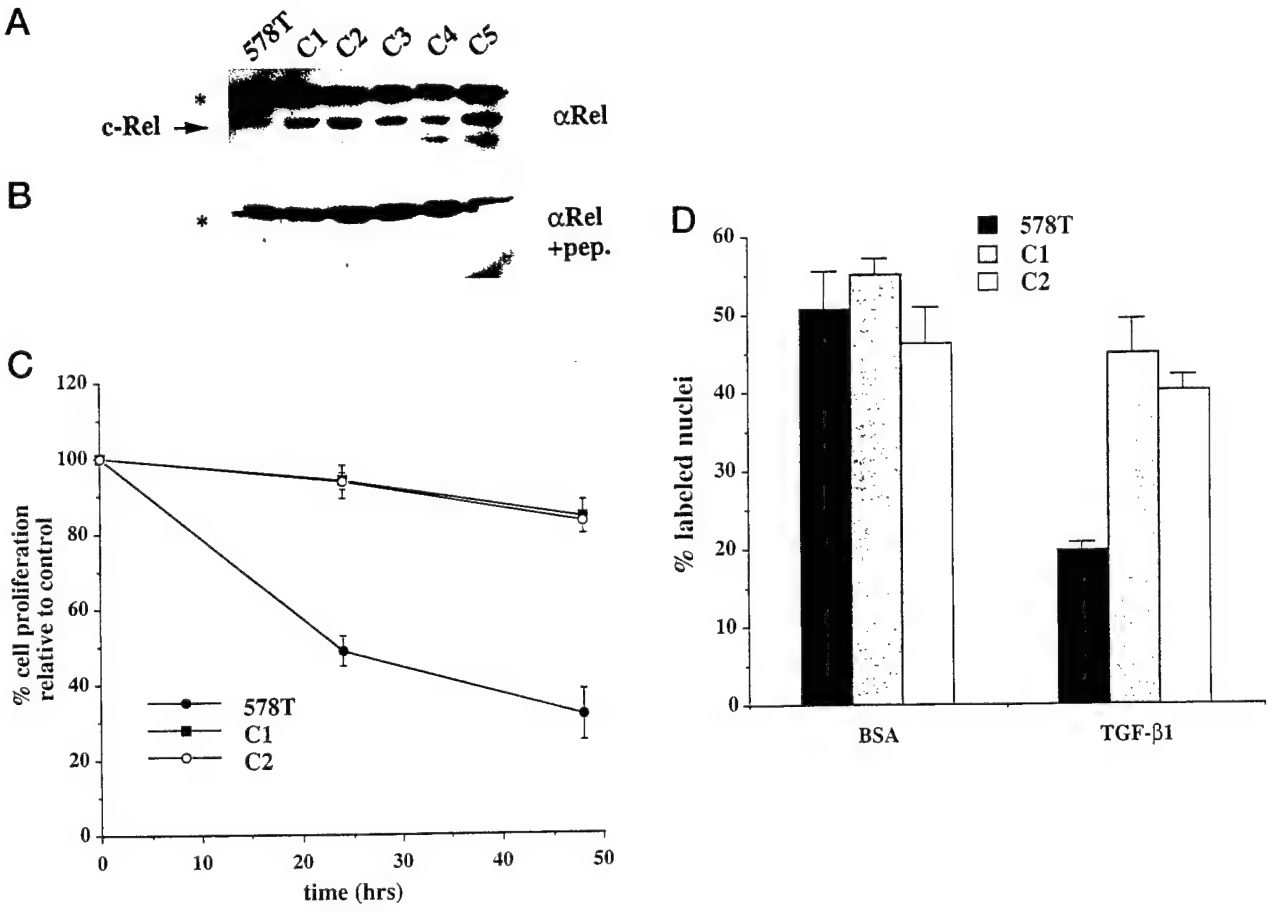


Fig. 5. Ectopic c-Rel expression in individual clones ablates TGF-β1-mediated growth inhibition. Individual clones (*C1–C5*) were isolated from the Hs578TR mixed population by limiting dilution. *A* and *B*, immunoblot analysis. Expression of c-Rel in the individual clones was determined by immunoblot analysis. Nuclear extracts were isolated from exponentially growing clones and from parental Hs578T cells, and equal samples (80 μg) were subjected to immunoblot analysis using an anti-c-Rel antibody (SC-070, Santa Cruz Biotechnology) in the absence (*A*) or presence (*B*) of a 1:1 molar ratio of cognate peptide. *, position of a nonspecific band. *C*, MTS proliferation assay. Parental Hs578T and clonal Hs578TR-C1 and Hs578TR-C2 cells were plated at 20% confluence and treated with 5 ng/ml TGF-β1 or BSA as control for 24 and 48 h. The effects of TGF-β1 on growth were measured by MTS assay. Cell numbers for TGF-β1-treated cells are given as percent values relative to BSA-treated control cells. *D*, DNA synthesis. Parental Hs578T and clonal Hs578TR-C1 and Hs578TR-C2 cells were treated in duplicate with 5 ng/ml TGF-β1 for 48 h or with BSA as control. Cells were then incubated in media containing 2 μCi of [^{3}H]thymidine per ml for 6 h, fixed, and exposed for autoradiography. Percent labeled nuclei was determined by visual counting. Mean and SD were determined in two different experiments. Black columns, parental Hs578T cells; grey columns, clone 1; white columns, clone 2.

expression of p65. These results, taken together with the findings presented above, indicate that expression of either RelA or c-Rel can significantly reduce the growth-inhibitory activity of TGF-β1 on Hs578T breast cancer cells.

Discussion

Here we provide evidence that the drop in NF-κB/Rel plays an important role in TGF-β1-mediated inhibition of breast cancer cell growth. TGF-β1 treatment of Hs578T and MCF7 human breast cancer cell lines decreased their rate of proliferation and concomitantly decreased the overall levels of NF-κB/Rel binding activity in these cells. Ectopic expression of either c-Rel or p65 led to resistance to the growth inhibitory effects of TGF-β1, demonstrating a direct role of NF-κB/Rel factors in control of proliferation. Stabilization of IκB-α specifically was implicated in the observed decrease in NF-κB/Rel activity. Overall, these studies indicate that NF-κB/Rel activity is important in the control of breast tumor cell proliferation. Previously, activation or constitutive NF-κB/Rel expression had been reported to promote growth of various cell types, including B and T lymphocytes, fibroblasts, and smooth muscle and liver cells (20, 21, 30–35). Our results extend the growth-promoting role of NF-κB/Rel factors to neoplastically transformed breast epithelial cells; furthermore, they suggest that targeting this activity may be useful in the treatment of breast cancer.

The inhibitory effects of TGF-β1 on breast cancer have been demonstrated *in vivo* with transgenic mouse studies. MMTV-TGF-β1 transgenic mice were highly resistant to DMBA-induced tumorigenesis, suggesting that overexpression of TGF-β1 has profound inhibitory effects on breast cancer development (6). The resistance of the MMTV-TGF-β1 mice to DMBA suggests that TGF-β1 counteracts the actions of DMBA. It is possible that developmental ef-

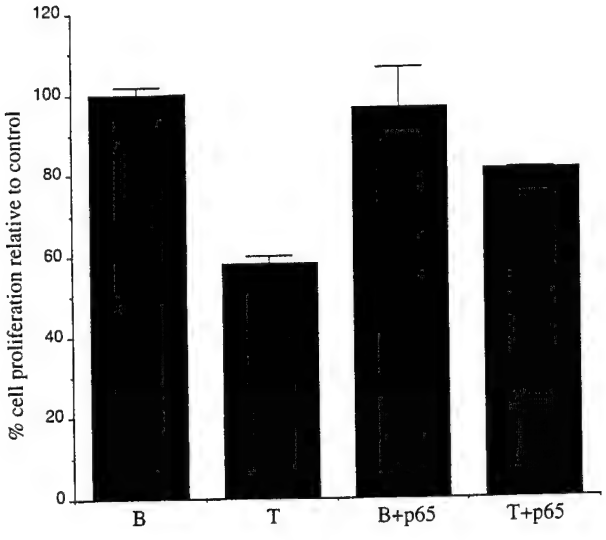


Fig. 6. Ectopic expression of p65 ablates TGF-β1-mediated growth arrest of Hs578T cells. Hs578T cells were plated in triplicate at 70% confluence in 96-well dishes. After removal of the media, cells were incubated according to the manufacturer's directions for 24 h in a 4-μl solution of DNA in FUGENE [either 130 ng pMT2T parental + 20 ng GFP/well, or 130 ng of human p65 pMT2T + 20 ng GFP/well (+*p65*)]. After 24 h, the cells were treated with carrier BSA (*B*) or with 5 ng/ml TGF-β1 (*T*), and the effects of TGF-β1 on growth were measured by MTS assay. The average of two experiments are shown; values are given as percent cell proliferation relative to BSA carrier-treated control cells that had been transfected with the parental pMT2T vector DNA. Transfection efficiency was estimated to be approximately 70% based on GFP staining.

fects of TGF-β1 expression decrease the mammary gland susceptibility to DMBA-induced tumorigenesis. However, the glands in these animals were still able to differentiate normally during pregnancy, which indicated that development of the gland was not severely impaired. Interestingly, we have recently shown that mammary tumors induced in female Sprague-Dawley rats treated with DMBA are typified by aberrant induction of NF-κB/Rel activity (26). Specifically, we observed that over 85% of tumors expressed high levels of nuclear NF-κB/Rel. On the basis of the work presented here, it is tempting to speculate that inhibition of NF-κB/Rel activity may play an important role in the ability of TGF-β1 to interfere with the DMBA-induced tumorigenic process.

Zugmaier *et al.* (8) studied the effects of TGF-β1 on numerous breast cancer cell lines and were able to inhibit the growth of most cell lines independent of ER status. However, there was a discrepancy in the response of MCF7 cells depending on passage number. Early passage (<100) MCF7 cells were inhibited by TGF-β1, while late passage (>500) cells were resistant to its effects (data not shown), which suggests that biological changes that occur with continuous passage *in vitro* are in part responsible for the variable phenotype of this cell line. The MCF7 cells used in these experiments were above passage number 154 (as provided by the ATCC) and were still sensitive to the growth-inhibitory effects of this agent.

Although there are published reports describing MCF7 cells undergoing apoptosis after treatment with TGF-β1 (10, 36), several other studies did not observe apoptosis of breast cancer cells on TGF-β1 treatment (8). In our experiments, TGF-β1 did not appear to induce apoptosis in either MCF7 or Hs578T cells, as judged by propidium iodide staining of chromatin, DNA laddering, or terminal deoxynucleotidyl transferase mediated nick end labeling assay. In our previous study (26), apoptosis was induced in 40% of Hs578T cells microinjected with IκB-α. There are several explanations of the apparent lack of cell death in the present studies. It is possible that partial inhibition of NF-κB/Rel slows cellular growth, and that complete and rapid inhibition is necessary to induce apoptosis. Interestingly, residual NF-κB/Rel binding levels was observed in both of the cell types after TGF-β1 treatment. Also, similar to differential sensitivity to growth inhibitors (8), sensitivity to the apoptotic effects of TGF-β1 may change with passage number. Alternatively, cells may be differentially sensitive to apoptotic stimuli depending on the stage of the cell cycle.

The studies presented here indicate that the inhibition of constitutive NF-κB/Rel activity may not be sufficient to induce apoptosis in all breast cancer cells; however, downregulating this activity may decrease proliferation of breast tumor cells. Several studies have found an association between response to TGF-β1 and poor prognosis in patients (36–40). The findings raised the question of whether the loss of responsiveness to TGF-β1 is associated with a progression to more aggressive breast tumors. Our findings raise the intriguing possibility that resistance to TGF-β1, which is most often due to the loss of TGF-β1 receptor (27, 41–43), can be circumvented inasmuch as the same inhibitory effects on tumor cell proliferation may be achieved via the use of inhibitors of NF-κB. Thus, inhibition of NF-κB may provide a new method to sensitize breast cancer cells to chemotherapeutic treatments.

Materials and Methods

Cell Lines Culture Conditions and Treatments. MCF7 cells were kindly supplied by F. Foss (Boston University Medical School, Boston, MA) and C. Sonnenschein (Tufts University Medical School, Boston, MA), or purchased from the ATCC. Human breast cancer Hs578T cells were kindly supplied by M. Sobel (National Cancer Institute, Bethesda, MD), or purchased from the ATCC. MCF7 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS (Life Technologies, Inc., Gaithersburg, MD), 100 μg/ml streptomycin (Life Technologies, Inc.), and 100 units/ml penicillin (Life Technologies, Inc.). Hs578T cells were propagated in DMEM with 10% heat-inactivated FCS, 4.5 g/liter glucose, 10 μg/ml of insulin (Sigma Chemical Co.), and antibiotics as above. Cells were plated the day before treatment: Hs578T cells at densities ranging from 2,600–4,300 cells/cm^2 and MCF7 cells at 7,100–14,000 cells/cm^2 depending on the length of treatment. A sterile stock solution of TGF-β1 (R&D Systems, Minneapolis, MN) was made to 5 ng/μl dissolved in 0.1% carrier BSA solution. The final concentration of TGF-β1 in the media culture was 1–5 ng/ml as indicated; controls were treated with equal amounts of BSA carrier solution.

For the analysis of cells entering DNA synthesis, the percentage of cells incorporating [^{3}H]thymidine was measured. Briefly, cells were labeled for 6 h with 2 μCi/ml and fixed, and the percentage of labeled nuclei was assessed, as we have described previously (44). For the Non-radioactive Cell Proliferation assay (Promega), cells were seeded at the indicated confluence in 96-well tissue culture dishes. TGF-β1- and BSA-treated cultures were incubated in triplicate for 4–6 h in the presence of MTS tetrazolium salt compound solution (333 μg/ml) and 25 μM phenazine methosulfate according to the manufacturer's directions. The A490 was measured using an ELISA plate reader. For studies on the half-life of IκB-α, cells were plated the previous day to achieve 50% confluency on

the day of treatment (Hs578T cells, 2×10^5/p100; MCF7, 5.2×10^5/p60). A stock solution of 20 mg/ml of emetine (Sigma) was made in water, and cells were treated with 20 μg/ml for various lengths of time as indicated.

EMSA. Nuclear extracts were prepared from breast cancer cells by a modification of the method of Dignam *et al.* (45) Cells were washed twice with ice-cold PBS (Ca^{2+}- and Mg^{2+}-free) containing protease inhibitors (0.5 mM DTT, 0.5 mM PMSF, and 10 μg/ml LP). They were then resuspended in 1 ml of cold hypotonic RSB buffer [10 mM NaCl, 3 mM $MgCl_2$, and 10 mM Tris (pH 7.4)] containing 0.5% NP40 detergent plus protease inhibitors as above. After a 15 min incubation on ice, the cells were dounce-homogenized until cell lysis occurred. Nuclei were resuspended in two packed nuclear volumes of extraction buffer C plus protease inhibitors as above and incubated on ice for 30 min. Protein concentration was determined using the Bio-Rad protein assay, following the manufacturer's directions. For the labeling of the NF-κB URE or Oct-1 oligonucleotides, a 150–300-ng sample was incubated for 30 min at 37°C in a solution adjusted to a final concentration of 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 μM each dATP and dTTP, 50 μCi each of [^{32}P]dCTP and [^{32}P]dGTP, and 5 units of Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA). The URE oligonucleotide, described previously (29), has the following sequence: 5′-GATCCAAGTCCGGGTTTTCCCCAA CC-3′. The underlined sequences indicate the core binding elements. The Oct-1 oligonucleotide has the following sequence: 5′-TGTCGAATGCAAATCACTAGAA-3′. For the binding reaction, ^{32}P-oligonucleotide (20,000–25,000 cpm) was incubated with 5 μg of nuclear extract, 5 μl sample buffer (10 mM HEPES, 4 mM DTT, 0.5% Triton X-100, and 2.5% glycerol), 2.5 μg poly dI-dC as nonspecific competitor, and the salt concentration adjusted to 100 mM using buffer C. The reaction was carried out at room temperature for 30 min. DNA/protein complexes were subjected to electrophoresis at 11 V/cm and resolved on a 4.5% polyacrylamide gel (using 30% acrylamide/0.8% bisacrylamide) with 0.5× TBE running buffer [90 mM Tris, 90 mM boric acid, and 2 mM EDTA (pH 8.0)].

Immunoblot Analysis. For cytoplasmic and nuclear extracts, cells were washed twice with PBS containing DTT, PMSF, and LP, as described above; resuspended in 200–400 μl lysis buffer [10 mM Tris (pH 7.6), 10 mM KCl, and 5 mM $MgCl_2$] containing the above protease inhibitors and 1% NP40; and incubated in ice for 5 min. Nuclei were pelleted for 4 min at 2,500 rpm at 4°C. The supernatant containing cytoplasmic proteins was stored at −80°C. The nuclear pellet was washed once in lysis buffer without detergent, centrifuged, and the nuclear proteins extracted using 100–300 μl radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium lauryl sarcosine, 1% NP40, 0.1% SDS, and 1 mM EDTA] plus DTT, PMSF, and LP. The DNA was sheared by pulling the solution 20 times, first through a 23G and then a 25G7/8 needle. After microcentrifugation for 30 min at 14,000 × *g* at 4°C, the supernatant containing the nuclear proteins was removed and stored at −80°C. Protein concentrations were determined using the Bio-Rad *Dc* protein assay. Proteins samples (20–40 μg) were resolved in a 10% polyacrylamide-SDS gel, transferred to PVDF membrane (Millipore, Bedford, MA), and subjected to immunoblotting, as described previously (26). The antibodies preparation for IκB-α (SC-371), IκB-β (SC-945), and c-Rel (SC-070) were purchased from Santa Cruz Biotechnology Inc.

Isolation of Hs578T Stable Transfectants. Activity of the murine c-Rel expression vector pSV-SPORT-c-Rel (kindly provided by T. Gilmore, Boston University, Boston, MA), which encodes a full-length c-Rel protein, was confirmed by transient transfection analysis in 3T3 cells, which do not express constitutive NF-κB/Rel factors (46).[4] The Hs578T c-Rel stable transfectants were prepared using 38 μg of pSV-SPORT-c-Rel and 2 μg of pSV_2neo DNA. Cells were transfected by calcium phosphate as described previously (26). After 24 h, 1.2 mg/ml G418 (Life Technologies, Inc.) were added to the medium, and selective growth conditions were maintained for approximately 2 weeks. Clones were isolated by limiting dilution.

Transient Transfection of Hs578T Cells. Hs578T cells were plated in triplicate at 70% confluence in 96-well dishes. After removal of the media, cells were incubated for 24 h in a 4-μl solution of DNA in FUGENE Transfection reagent (Boerhinger/Mannheim), according to the manufacturer's directions. DNA used per well was either 130 ng of human p65 pMT2T (kindly provided by U. Siebenlist, NIH, Bethesda, MD) or parental pMT2T DNA, plus 20 ng of GFP expression plasmid (kindly provided by C. Gelinas, Robert Wood Johnson Medical School, Piscataway, NJ). After 24 h, the cells were treated either with carrier BSA or with 5 ng/ml TGF-β1, and the effects on growth were measured by MTS assay after 48 h. An approximate 70% transfection efficiency was estimated using GFP staining.

[4] D. W. Kim and G. E. Sonenshein, unpublished observations.

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Carcinogenesis

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January 31, 2000

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Dear Dr. Sonenshein,

I am pleased to inform you that your manuscript "Activation of NF-kB/Rel occurs early during neoplastic transformation of mammary cells" is acceptable for publication in *Carcinogenesis* and should appear in a forthcoming issue. Thank you for submitting your interesting work to *Carcinogenesis*.

Sincerely,

C.C. Harris jp

Curtis C. Harris
Executive Editor

Activation of NF-κB/Rel Occurs Early During Neoplastic Transformation of Mammary Cells

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Abstract

NF-κB/Rel is a family of transcription factors, which are expressed in all cells; however, in most non-B cells, they are sequestered in the cytoplasm in inactive complexes with specific inhibitory proteins, termed IκBs. We have recently shown that NF-κB/Rel factors are aberrantly activated in human breast cancer and rodent mammary tumors, and function to promote tumor cell survival and proliferation. Here, we have examined the time course of induction of NF-κB/Rel factors upon carcinogen treatment of female Sprague-Dawley (S-D) rats *in vivo* and in human mammary epithelial cells (HMECs) in culture. We observed that NF-κB/Rel activation is an early event, occurring prior to malignant transformation. In S-D rats, increased NF-κB/Rel binding was detected in nuclear extracts of mammary glands from 40% of animals 3 weeks post treatment with 15 mg/kg 7,12-dimethylbenz(*a*)anthracene (DMBA); this is prior to formation of tumors which normally begin to be detected after 7 to 9 weeks. In non tumorigenic MCF-10F cells, *in vitro* malignant transformation upon treatment with either DMBA or benzo[*a*]pyrene (BaP) resulted in a 4- to 12-fold increase in activity of classical NF-κB (p65/p50). NF-κB induction was corrrelated with a decrease in the stability of the NF-κB specific inhibitory protein IκB-α. Ectopic expression of the transactivating p65 subunit of NF-κB in MCF-10F cells induced the c-*myc* oncogene promoter, which is driven by two NF-κB elements, and endogenous c-Myc levels. Furthermore, reduction mammoplasty-derived HMECs, immortalized following BaP exposure, showed dysregulated induction of classical NF-κB prior to malignant transformation. Together these findings suggest that activation of NF-κB plays an early, critical role in the carcinogen-driven transformation of mammary glands.

Introduction

NF-κB/Rel is a family of dimeric transcription factors distinguished by the presence of a Rel homology domain (RHD) of about 300 amino acids in length which determines much of its function. Classical NF-κB is a heterodimer composed of p65 and p50 subunits (1). Other members of the mammalian Rel family include c-Rel, p52 (also called lyt10), and RelB. The p65 and RelB, and c-Rel subunits have either potent or moderate transactivation potential, respectively, whereas, the p50 and p52 subunits bind avidly, but have only modest transactivation abilities (1). In most cells, other than B lymphocytes, NF-κB/Rel proteins are sequestered in the cytoplasm bound to one of the specific inhibitory proteins termed IκB's of which IκB-α is the paradigm. A variety of agents can induce NF-κB/Rel (2). Activation of NF-κB involves phosphorylation, followed by ubiquitination and proteosome-mediated degradation of IκB, which allows for translocation of active NF-κB complex into the nucleus where it can bind to κB responsive elements (2).

NF-κB/Rel has now been established as a factor promoting survival from apoptosis (3). An anti-apoptotic function for constitutively expressed NF-κB/Rel factors has been demonstrated in several cell types, including B lymphocytes (4,5), and hepatocytes (6,7). Several groups also found that induction of NF-κB/Rel upon treatment with Tumor Necrosis Factor (TNF)-α, radiation, and chemotherapeutic agents can protect cells from apoptosis (8-11). More recently, we showed that aberrant activation of NF-κB/Rel occurs in human breast cancer cells and that specific inhibition of this activity leads to the induction of apoptosis (12). There is also increasing evidence that NF-κB/Rel family is important in control of cell proliferation and oncogenesis, e.g. correlation of factor activation has been reported in various types of cancer (13).

It has been suggested that some of the rise in breast cancer rates reflects increased exposure to and bioaccumulation of lipophilic environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) like benzo[*a*]pyrene (BaP), and related organochlorines (14). This hypothesis has been drawn, in part, from epidemiologic studies associating elevated breast cancer rates with PAH exposure (15-18) and from studies demonstrating increased levels of aromatic hydrocarbons in breast carcinomas (19,20), and in sera from breast cancer patients (18). Furthermore, many studies have shown that PAHs can cause malignant transformation of rodent

models *in vivo* (21) and human mammary epithelial cells (HMECs) *in vitro* (22). For example, in Sprague-Dawley (S-D) rats, a single intragastric dose of DMBA induces mammary tumors within 7-20 weeks (21). While much has been learned about the effects of carcinogen treatment on gene activation and DNA adduct formation (23), the exact molecular mechanism(s) by which this transformation occurs has yet to be elucidated. Recently, we observed that NF-κB/Rel factors are aberrantly activated in 86% of the mammary tumors induced by DMBA-treatment of S-D rats compared to the normal mammary glands of the age-matched, vehicle-treated control animals (12). Here, we have examined the time course of carcinogen-mediated induction of NF-κB/Rel using the S-D rat model and the *in vitro* treated HMECs. We show that NF-κB/Rel activation occurs prior to malignant transformation, suggesting it plays an early and potentially important event in the progression of breast epithelial cells towards a malignant phenotype.

Materials and Methods

Cell Growth and Treatment Conditions.

Hs578T tumor cell line was derived from a carcinosarcoma and is epithelial in origin (24). MCF-10F (HMECs) were established from a patient with fibrocystic disease and do not display characteristics of a malignant phenotype (22). These cells represent a non-tumorigenic, immortally transformed cell line. The D3-1 and BP-1 lines were derived by DMBA- and BaP-mediated transformation of MCF-10F cells, respectively (22), and were cultured as published previously (22). 184 HMEC strain, derived from reduction mammoplasty tissue, and the 184A1 cell line, which emerged from 184 HMEC after exposure to BaP, were cultured as described (25). These HMEC were arrested in G0 by exposure for 48 hours to medium lacking EGF and containing the anti-EGF receptor antibody, Mab 225, as described (26). For IκB-α protein turnover assays, exponentially growing cells were treated with 20 ug/ml emetine (Sigma Chemical Co., St. Louis, MO) and cytoplasmic extracts (50 ug/sample) subjected to immunoblot analysis with the IκB-α antibody (sc# 371, Santa Cruz Biotechnology, Santa Cruz CA), essentially as previously described (12).

Electric Mobility Shift Analysis (EMSA).

Nuclear extracts were prepared from breast cancer cells essentially as describe previously (12). Briefly, cells were washed twice with ice cold PBS (Ca^{2+} and Mg^{2+} free) containing protease inhibitors (0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10

ug/ml leupeptin (LP)). They were then resuspended in 1 ml of cold hypotonic RSB buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris pH 7.4) containing 0.5% NP-40 detergent plus protease inhibitors as above. Following a 15 minute incubation on ice, the cells were dounce homogenized until cell lysis occurred. Nuclei were resuspended in 2 packed nuclear volumes of extraction buffer C (420 mM KCl, 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol) plus protease inhibitors as above, and incubated on ice for 30 min. Protein concentration was determined using the Bio-Rad protein assay, following the manufacturer's directions (Bio-Rad Laboratories, Hercules, CA). The sequence of the NF-κB-containing oligonucleotide from the c-*myc* gene (27) is as follows: 5'-GATCCAAGTCCGGGTTTTCCCCAA CC-3', where the underlined region indicates the core binding element. The sequences of the PU.1- and TCF-1-containing oligonucleotides are as follows, PU.1: 5'-GATCTACTTCTGCTTTTG-3'; TCF-1: 5'-GGGAGACTGAGAACAAAGCGCTCTCACAC-3' (28). For labeling of the double-stranded oligonucleotide, which has 5' overhangs allowing fill-in with DNA polymerase I, a 150-300 ng sample was incubated for 30 min at 37°C in a solution adjusted to a final concentration of 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 uM each of dATP and dTTP, 50 uCi each of [^{32}P]-dCTP and [^{32}P]-dGTP, and 5 units of Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA). For the binding reaction, ^{32}P-labeled oligonucleotide (20,000 - 25,000 cpm) was incubated with 5 ug of nuclear extract, 5 ul sample buffer (10 mM HEPES, 4 mM DTT, 0.5% Triton X-100, and 2.5% glycerol), 2.5 ug poly dI-dC as nonspecific competitor, and the salt concentration adjusted to 100 mM using buffer C. The reaction was carried out at room temperature for 30 min. DNA/protein complexes were subjected to electrophoresis at 11 V/cm and resolved on a 4.5% polyacrylamide gel (using 30% acrylamide/0.8 % bisacrylamide) with 0.5x TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0). Supershift analysis were performed by incubating with the appropriate antibodies for 1 additional hour after the binding reaction had taken place. For supershift/blocking analysis, either of two antibodies against the p50 subunit were used; these were either kindly provided by K. LeClair (Procept, Inc., Cambridge MA) or purchased (sc-114, Santa Cruz Biotechnology). Antibodies against the p65 and c-Rel subunits were sc-109 and sc-372, Santa Cruz Biotechnology and #1226, kindly provided by N. Rice (NCI, Frederick MD), and sc-070 (Santa Cruz Biotechnology), respectively.

Transfection and Transactivation Analysis.

Wildtype (E8) and mutant (mutE8) NF-κB element-thymidine kinase (TK) promoter-chloramphenicol acetyltransferase (CAT) reporter vectors were constructed as reported previously (27). Briefly, these consisted of 2 copies of either the wild type NF-κB element from upstream of the c-*myc* promoter, sequence given above, or versions with the two internal G residues converted to C residues, which significantly reduces NF-κB binding and transactivation (27). Each cell type displayed very large differences in transfection efficiency, and thus optimization of the specific transfection protocol was performed for each individual line. D3-1 cells were transfected by a modified calcium phosphate protocol, as described previously (12). MCF-10F and BP-1 cells were transfected using Lipofectamine reagent (Gibco BRL, Gaithersburg, MD). Cultures of 184 and 184 A1 cells were transfected using Cytofectin (Glen Research, Sterling, VA). All cells were harvested using reporter lysis buffer (Promega, Madison, WI) and CAT assays and luciferase assays were performed as published previously (12). Alternatively, confluent cultures of MCF-10F cells were transiently transfected the p1.6 Bgl c-*myc* promoter CAT, containing 1.2 kB of upstream and 0.4 kB exon 1 sequences, including the two NF-κB elements (29), using FUGENE transfection reagent (Boehringer Mannheim, Indianapolis, IN), according to the manufacturer's instructions. Vector *pEVRF-p65*, encoding murine p65 protein (kindly provided by R. Sen, Brandeis University, Waltham MA), was co-transfected, as indicated. Total DNA transfected was maintained at either 4-5 μg. Cells were harvested after 24 h, and extracts normalized for protein assayed, as above.

Transfection and Immunoblot Analysis.

Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4 μg *pEVRF-p65* plus 20 μg *pcDNA3* plasmid or with 24 μg *pcDNA3* plasmid DNA with 30 μl FUGENE transfection reagent. After 48 h, cells were rinsed with cold PBS, and harvested in lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 150 mM NaCl; 0.5 mM DTT; 2 μg/ml aprotinin; 2 μg/ml leupeptin; 0.5 mM PMSF; 0.5% NP40). Whole cell extracts (WCE) were obtained by sonication, followed by centrifugation at 14,000 rpm for 30 min. Samples (40 μg) of WCEs were subjected to electrophoresis and immunoblot analysis, as above. Blots were probed with rabbit anti-c-Myc antibody (786-4, a gift from S. Hann, Vanderbilt University, Memphis TN), and mouse anti-β-actin monoclonal antibody (AC-15, Sigma).

Carcinogen Treatment of Rats.

Virgin female S-D rats, fed AIN-76A diet, were handled according to a protocol approved by the Boston University Institutional Animal Care and Use Committee. For analysis of mammary glands from normal animals, 5 virgin female S-D rats (Charles River Laboratories, Wilmington, MA), 6 weeks of age, were housed in the AAALAC-approved Laboratory Animal Science Center and fed AIN76 diet for 3 weeks. They were euthanized with CO_2; all 12 mammary glands were combined for each animal, and nuclear extracts prepared as described previously (12). For the time course analysis, S-D rats from the same supplier were housed in environmentally controlled animal quarters in the Mallory Institute of Pathology, fed AIN-76A diet, and randomly entered into control or DMBA-treated groups of either 4 or 5 animals. At age 8 weeks they were given either a single intragastric dose of 15 mg/kg DMBA dissolved in sesame oil or given the sesame oil vehicle alone. Rats were predesignated to be killed and necropsied at 6 or 24 hours, or 1, 3 or 9 weeks after DMBA or oil administration. Rats were palpated weekly for tumor. At necropsy of each control and DMBA-treated rat, all 12 mammary glands were rapidly removed, combined, and frozen in liquid nitrogen for storage. At nine weeks two rats were found to have non-palpable tumors in one gland each. These tumors were excluded from the mammary gland samples taken for the time course study. Nuclear extracts were prepared, as described previously (12), and subjected to EMSA.

Results

Activation of NF-κB/Rel Occurs Prior to Tumor Formation in Mammary Glands of Rats Treated with DMBA.

Recently, we have demonstrated that 86% of the mammary tumors induced by DMBA-treatment of S-D rats displayed aberrant activation of NF-κB/Rel compared to the normal mammary glands of the age-matched, vehicle-treated control animals (12). To determine the nature of the NF-κB binding activity in the normal mammary gland, EMSA was performed on nuclear proteins isolated from the mammary glands of 5 virgin female 9 week old S-D rats and a radiolabelled NF-κB-containing oligonucleotide, as probe (Fig. 1A). A major band (Band 1) was seen (Fig. 1A), which was better resolved on a light exposure (Fig. 1B); many extracts also displayed a minor slower migrating complex (Band 2) seen on a longer exposure (Fig. 1A and data not shown). To identify the nature of the subunits within these complexes, antibody

supershift/blocking EMSA was performed. Addition of a supershifting antibody against the p50 subunit of NF-κB yielded a new band and dramatically reduced formation of the Band 1 complex (Fig. 1B). Addition of a blocking antibody against p65 ablated formation of Band 2 (Fig. 1C). Addition of an antibody against c-Rel had no effect on binding (data not shown). From these experiments, we conclude that normal mammary glands express predominantly p50 homodimers with minor amounts of a p65-containing complex. Based on its migration, Band 2 likely represents p50/p65 heterodimers.

We performed a time course experiment to assess the kinetics of activation of NF-κB/Rel in S-D rats as a function of carcinogen treatment (12). S-D rats were administered 15 mg/kg DMBA in sesame oil by intragastric gavage. At this dose, tumors are first detectable after 7 weeks, and numbers increase until approximately 90% of animals have tumors at 15 weeks (21). Control rats were similarly administered the vehicle. Animals were necropsied at 6 hours, 24 hours, 1 week, 3 week, and 9 weeks. For each animal, all 12 mammary glands were excised, combined and nuclear extracts prepared. (At the 9 week time point, a tumor was observed in one mammary gland of each of two DMBA-treated animals; these were excluded from the remaining glands which were histologically normal). EMSA analysis was performed using a radiolabelled NF-κB-containing oligonucleotide as probe with extracts from treated or control animals (Figure 2A and 2B, respectively). A sample of a nuclear extract from an S-D rat mammary tumor, with a typical level of binding, was added to each gel to permit comparison of the relative levels of NF-κB/Rel expression amongst the various samples. As seen previously (12), NF-κB/Rel binding levels were extremely low in mammary gland extracts from control, vehicle-treated rats after a 6-hour period (Figure 2B), consistent with data presented in Fig. 1A. Similar binding levels were seen with additional control animals, e.g., after various times up to the 9-week period (data not shown). Overall, normal mammary glands exhibited less than 10% of the binding levels seen in the tumor sample, as judged by densitometry. After 6 hours, 24 hours or 1 week following DMBA treatment, no increase in NF-κB/Rel binding was detected. However, at 3 weeks, there was clearly significant activation of formation of all complexes in two of the five animals (#46 and #47) that were administered DMBA. The binding levels in these animals approached 39% and 48% of the control tumor sample, as judged by densitometry. At nine weeks, in two of the five animals (#53and #55) induction of higher constitutive levels of NF-κB/Rel was again detected. Interestingly, these rats were the ones discussed above that had developed one

mammary tumor each. The higher intensity of binding in these four samples was not due to unequal loading, as judged by EMSA with an Sp1 oligonucleotide (data not shown). Also no contamination of B lymphocytes, neutrophils, mast or myeloid cells, or of T lymphocytes was detected as judged by EMSA with oligonucleotides containing binding sites for PU.1, and TCF-1 (Figs. 2C and 2D, respectively, and data not shown). Thus, these data indicate that activation of NF-κB/Rel binding in the mammary gland can occur 3 weeks post DMBA administration, preceding tumor formation which is not detectable prior to 7 weeks of treatment with this dose of carcinogen (21) and was not detected at necropsy until 9 weeks in this experiment.

Transformation of MCF-10F Cells by the Polycyclic Aromatic Hydrocarbons DMBA and BaP Increases NB-κB Activity.

We next sought to determine whether treatment with PAHs *in vitro* would similarly lead to an induction of NF-κB/Rel activity in HMECs. D3-1 and BP-1 cell lines were derived from the non-tumorigenic MCF-10F cell line by 24 hour treatment of either DMBA and BaP, respectively. Both cell lines exhibit malignant characteristics. BP-1 cells exhibit increased anchorage independent growth, increased chemotaxis and chemoinvasiveness. D3-1 cells exhibit increased chemotactic and chemoinvasive capabilities, but to a lesser extent than BP-1 cells (22). Nuclear extracts from D3-1 and BP-1 cells displayed significantly increased NF-κB binding activity compared to the parental MCF-10F cells (inset Figure 3A). Equal loading was confirmed in EMSA using an Oct-1 probe (data not shown). Two bands were seen with the extracts from the D3-1 and BP-1, which co-migrated with bands seen with nuclear extracts from the MCF-10F cells. (The upper band with MCF-10F extract was better seen on a longer exposure.)

To confirm that the binding activity was functional, transient transfection analysis was performed using E8 and mutE8 multimerized NF-κB element-driven TK promoter-CAT reporter constructs. Since transfection efficiency of these lines varied, the activity of wild type κB construct was normalized to that of the mutE8 activity, which is reflective of basal reporter activity without any contribution from NF-κB. The parental MCF-10F cells showed a minimal induction of E8 activity over the mutE8 of approximately 1.7-fold +/- 0.6 (Figure 3A). The D3-1 and BP-1 cells showed a significantly higher level of NF-κB activity of 4.1-fold +/- 1.4 and 11.6-fold +/- 0.2, respectively. Thus, the relative levels of binding and activity correlate directly.

To confirm that the activity of the E8 vector seen in the transformed cell lines was due to NF-κB/Rel binding, co-transfection analysis was performed with an increasing dose of a vector

expressing the NF-κB/Rel specific inhibitory protein IκB-α. The activity of the E8 vector in the D3-1 cells was specifically repressed in a dose-dependent manner by IκB-α (Figure 3B). Similarly, co-transfection with 1.4 ug of vector expressing IκB-α reduced NF-κB-specific activity 3.6-fold +/-0.1 in the BP-1 cells. Thus, the transformed D3-1 and BP-1 cell lines display increased levels of functional NF-κB/Rel than seen in the parental MCF-10F cells.

Antibody supershift/blocking analysis was performed to identify the NF-κB subunit composition in the various extracts. Addition of an antibody against the p50 subunit to nuclear extracts from MCF-10F cells resulted in a supershift of the both bands, while addition of a blocking antibody against the p65 subunit deleted the upper complex selectively (Figure 4A). Therefore, the upper complex was identified as a p50/p65 complex, also known as classical NF-κB, a potent transactivator (1). Addition of an antibody to the p52 subunit had no effect on binding (data not shown). Thus, based on the faster migration of the bottom band, this complex likely consists of p50/p50 homodimers, which have only minimal transactivation potential. Similar analysis was performed with the D3-1 and BP-1 nuclear extracts (Figure 4B and data not shown). The upper band was eliminated upon addition of the p65 antibody with extracts from both cells, whereas, the p50 antibody completely shifted the lower band, and cleared most of the upper band as well (Figure 4B and data not shown). Addition of an antibody against c-Rel did not affect the pattern of migration with either cell extract. Thus, for the D3-1 and BP-1 cells, the upper band consists of the p50/p65 heterodimers, and the lower band consists of the p50/p50 homodimers, respectively. Thus, carcinogen transformation yields a functional binding complex of classical NF-κB at much higher intensity but of similar subunit composition as the parental MCF-10F cells, consistent with the identical patterns of migration.

IκB-α Has A Shorter Half Life In D3-1 And BP-1 Than In MCF-10F Cells.

In B cells, it has been shown that faster turnover of IκB-α proteins is, at least in part, responsible for the higher levels of functional NF-κB in the nucleus (30). Therefore, we next sought to determine if the increase in NF-κB levels in the transformed D3-1 and BP-1 cells could be correlated with changes in stability of the IκB-α protein. Exponentially growing cells were treated with emetine, a specific inhibitor of elongation of polypeptide chain synthesis, for 0, 1, 2, 3, 4, or 5 hours, and cytoplasmic extracts prepared and subjected to immunoblot analysis for IκB-α protein (Figure 5A). The decay of the IκB-α protein in the BP-1 and D3-1 cells appeared

more rapid than that observed in the MCF-10F cells. Equal loading of the lanes was confirmed by probing the same membranes with an antibody to the p65 subunit; this protein appeared to be quite stable even at 5 hours post treatment in all three cell types (data not shown). The resulting autoradiograms were quantified by densitometry and the data plotted in Figure 5B. The half-life of decay of IκB-α protein was determined in the BP-1 and D3-1 cells to be between 2-2.5 hours and 1-2 hours, respectively. In contrast, in the parental MCF-10F cells, a half life of approximately 7.8 hours was determined. Thus, the chemical transformation of these cells dramatically affects the stability of the IκB-α protein correlating with the higher levels of constitutively active NF-κB associated with the malignant phenotype.

Ectopic p65 Expression in MCF-10F Cells Induces the c-myc Oncogene

To begin to assess the potential functional role of the elevated levels of NF-κB activity following transformation of the MCF-10F, we performed transient transfection and measured the effects of increased p65 levels on expression of the c-*myc* oncogene, which contains two NF-κB elements (29). MCF-10F cells, in exponential growth, were transfected in duplicate with 0, 0.5 or 1 ug *pEVRF-p65* vector DNA expressing p65 protein and 1 ug p1.6 Bgl CAT c-*myc* promoter construct. An increase in c-*myc* promoter activity of 18.9 +/- 2.3-fold and 21.3 +/- 5.4-fold was detected with 0.5 and 1.0 ug p65 expression vector, respectively. To verify that p65 expression enhances the endogenous c-*myc* gene, similar transfections were performed. MCF-10F cells were transfected with *pEVRF-p65* vector DNA or with *pcDNA3* plasmid DNA. After 48 h, whole cell lysates were isolated and subjected to immunoblot analysis for levels of c-Myc and β-actin, as control for equal loading (Fig. 6). A significant increase in c-Myc expression was detected upon transfection of the *pEVRF-p65* vector DNA compared to vector DNA alone. In this and two duplicate experiments, a 3.4 +/- 0.7 fold increase was determined by densitometry. In contrast, levels of β-actin remained unchanged (Fig. 6). Thus, ectopic expression of the p65 subunit in MCF-10F cells leads to increased c-Myc levels.

HMEC Transformed by BaP Exhibit High Level of NF-κB Binding Activity Even in the Premalignant State.

To monitor the expression of NF-κB earlier during the transformation process, we compared the normal finite life-span 184 HMEC cell strain with its immortally transformed derivative cell line, 184A1. The 184 HMEC cell strain, derived from reduction mammoplasty tissue, senesce after approximately 80 population doublings. The immortal but non-tumorigenic 184A1 line was

established following BaP treatment of primary cultures of the 184 HMEC strain (25,31). NF-κB binding activity in the finite life-span 184 cells, and the late passage immortal 184A1 cell line was compared. Extracts from both cells gave a similar pattern with two bands detectable (Figure 7A), which co-migrated with the complexes observed in MCF-10F cells (data not shown). The levels of these complexes in the two populations of cycling cells were similar (Figure 7A). The half-life of decay of the IκB-α proteins in these cycling cell populations were also identical; a half-life of approximately 3.5 h was determined (data not shown). Previously, we had noted that BaP exposure led to dysregulated gene expression in quiescence (32). Thus, finite life-span and fully immortal cells were made quiescent upon blockage of EGF receptor signal transduction for 48 h, and nuclear extracts used in EMSA (Figure 7A). The levels of binding of finite life-span cells remained unchanged. In contrast, an increase in NF-κB binding activity was evident in the quiescent G0 immortally transformed 184A1 cells (Figure 7A). In fact, the level was essentially comparable to that found in the D3-1 and BP-1 lines; a commensurate decrease in half-life of decay of IκB-α was determined in these G0 cells to approximately 2.5 h (data not shown). Supershift EMSA was performed to identify the subunit components of the complexes in the cycling and quiescent 184A1 HMECs (Figure 7B). In nuclear extracts from 184A1 cells in either growth state, the upper complex was depleted upon addition of an anti-p65 antibody. Addition of an anti-p50 antibody abrogated formation of the bottom complex and reduced the upper complex. Thus in both cycling and arrested cells, the upper complex is classical NF-κB, composed of a p65 and p50 subunit, while the bottom complex is likely composed of a p50 homodimer. Similar complexes were identified with the 184 cell extracts (data not shown). These findings indicate that NF-κB is dysregulated in the growth-arrested late passage fully immortal 184A1 cells.

To assess the functionality of the increase in NF-κB binding in quiescence, transient transfection analysis was performed (Figure 8). Cells were transfected with E8 and mutE8, as above, and then incubated either in normal medium or deprived of EGF receptor signal transduction for to make them quiescent. As expected, when the finite life-span 184 HMECs cells were quiescent, the level of E8/mutE8 induction decreased from 6.1-fold to a 3.1-fold induction. The fact that the activity decreases most likely reflects the effects that quiescence has on reducing the overall rate of protein synthesis within these cells (26). When the immortally transformed 184A1 HMECs were similarly analyzed, an approximately 5-fold induction of

E8/mutE8 activity was seen in cycling cells, and this induction was maintained even when the cells were made quiescent (Figure 8). This maintenance of E8 activity is most likely due to the increased induction of NF-κB/Rel in the G0 state, and correlates with the increased binding displayed by EMSA (Figure 7A). Thus, even when growth arrested, NF-κB appears to maintain functionality in the fully immortal cells indicating a dysregulated expression of NF-κB in quiescence. Thus, dysregulation of expression of classical NF-κB can occur significantly prior to malignant transformation of the HMEC in culture.

Discussion

Here we demonstrate that the aberrant activation of NF-κB/Rel that typifies human and rodent breast cancer occurs early during the malignant transformation process, and involves de-stabilization of the specific inhibitory protein IκB-α. In S-D rats, activation of NF-κB/Rel occurred in the mammary glands of 40% of the animals 3 weeks after DMBA-treatment, a time point at which tumor formation was not observed. If not every mammary gland from a DMBA-treated animal has responded, this value potentially represents an underestimation due to the pooling of all 12 mammary glands from individual animals that was performed to facilitate the time course analysis. Furthermore, the observation of NF-κB/Rel activation *in vivo* with this rodent model was extended to cultured HMECs. Carcinogen induced transformation was found to result in elevated levels of NF-κB in the malignantly transformed D3-1 and BP-1 cells compared to their parental, non-malignant MCF-10F cells. This activation of classical NF-κB in the MCF-10F-derived lines correlated with de-stabilization of IκB-α. Furthermore, enhanced expression of p65 in MCF-10F cells led to induction of the c-*myc* oncogene. Lastly, dysregulated expression of NF-κB was seen in the immortal but non-malignant 184A1 cells compared to the finite life-span 184 strain. Given the involvement of NF-κB/Rel factors in control of genes signaling proliferation, cell survival and neoplastic transformation, the early and persistent functional activation of these factors in carcinogen-induced rodent and human mammary systems suggests a potential critical role in the malignant transformation process.

There is mounting evidence for NF-κB/Rel factors mediating signals that induce cell proliferation. Firstly, these factors have been found to transactivate critical genes controlling growth either directly, e.g. c-*myc* (27,29,33), or indirectly, e.g. various interleukins or growth

factors (1). Furthermore, constitutive NF-κB/Rel activity has been shown to be essential for proliferation of several cell types, e.g. smooth muscle cells (34), and hepatocytes during liver regeneration after partial hepatectomy or toxic damage (35). In T lymphoma cell line HuT 78, constitutive NF-κB activation, apparently due to autocrine TNF-α production, was shown to accompany enhanced cellular proliferation (36). Furthermore, we have recently found that ectopic expression of c-Rel subunit permits Hs578T breast cancer cells to overcome TGF-β1-induced growth inhibition (37). Consistent with this role of NF-κB/Rel, the D3-1 and BP-1 cells both proliferate at a faster rate than the parental MCF-10F cells (22), correlating well with the higher NF-κB activity in these cells. Likewise, the late passage 184A1 cells are able to proliferate indefinitely even in the presence of TGF-β1, compared to the parental 184 cells which are TGF-β1-sensitive.

Constitutive or induced activation of NF-κB has been implicated in promoting cell survival by protecting cells from undergoing apoptosis (3). Studies of mice null for the *RelA* gene, encoding the p65 protein, provided the first suggestive evidence (38). Deletion of the p65 subunit is embryonal lethal with liver degeneration due to extensive apoptosis of the hepatocytes (38). Direct evidence for the antiapoptotic role of NF-κB/Rel was demonstrated in breast cancer cells (12), B-lymphoma cells (3), and in hepatocytes (6): inhibition of constitutive NF-κB/Rel led to cell death by apoptosis. Furthermore, maintenance of NF-κB/Rel activity conferred protection from apoptosis induced by TNF-α or irradiation (8-11), TGF-β1 (7), or B cell receptor signals (4,39). In light of such evidence, the early activation NF-κB/Rel may play a similar role in the mammary gland following DMBA treatment leading to dysregulation of normal control of proliferation and protecting the epithelial cells from apoptosis.

Here we provide evidence that one of the genes regulated by NF-κB/Rel, that may promote epithelial cell proliferation and survival, is the c-*myc* proto-oncogene (40). A drop in c-*myc* has been found to induce apoptosis of many cell types including breast cancer cell lines (41), while conversely, c-*myc* overexpression is able to promote survival of several cell types, including B cells (40). Furthermore, overexpression of c-*myc* has been implicated in the etiology of breast cancer (42). The regulation of the c-*myc* promoter by NF-κB/Rel was first demonstrated in human breast cancer cells (12). Here, we extend this observation to MCF-10F cells showing that both the c-*myc* promoter and endogenous c-*myc* gene respond to ectopic

expression of p65. Interestingly, BP-1 cells overexpress the c-*myc* oncogene (43), and tumors from DMBA treated S-D rats have increased c-*myc* mRNA expression (D.W.K. and G.E.S., unpublished observations). Studies are in progress to determine whether inhibition of NF-κB/Rel activation leads to decreased c-*myc* gene expression and ablates the carcinogen transformation process.

Lastly, aberrant activation of nuclear NF-κB/Rel has been found to correlate with oncogenesis in several different systems, including breast cancer, thyroid carcinoma (44), non-small cell lung carcinoma, colon carcinoma, ovarian carcinoma, prostate cancer (45), Hodgkin's disease (46,47), and various types of lymphomas (13). The results presented here extend this association to carcinogen-induced *in vitro* malignant transformation of HMECs. Furthermore, our findings indicate that the activation of NF-κB/Rel in mammary glands upon carcinogen treatment of rodents is an early event. It was also interesting to note that in our studies with DMBA-treated S-D rats, the two animals studied at 9 weeks that displayed elevated NF-κB/Rel binding activity in the grossly normal mammary glands had non-palpable tumors. Taken together, our results suggest a novel mechanism for carcinogen induction of tumors of the mammary glands, i.e. via the activation of NF-κB/Rel factors. Thus, inhibition of NF-κB may provide a means of intervention at early, as well as later, stages of the transformation process in the mammary gland. Furthermore, the possibility that NF-κB/Rel may serve as a diagnostic marker for pre-malignant mammary glands warrants further investigation. Studies are underway to test directly the role of activation of NF-κB/Rel in neoplastic transformation by enforcing expression of a transactivating subunit of NF-κB/Rel in the mammary glands of transgenic mice.

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Figure Legends

Fig. 1. Nuclear extracts of normal mammary glands of female S-D rats express low levels of NF-κB complexes. **A)** EMSA was performed using samples (3 ug) of nuclear extracts from mammary glands of five S-D female rats (R1-R5), and an NF-κB oligonucleotide, as probe. Film was exposed for 7 days. The position of the major (Band 1) and minor (Band 2) complexes are indicated. The positions of the p50 homodimer and p50/c-Rel complexes formed with nuclear extracts of murine WEHI 231 B cells, isolated as described previously (4) and similarly analyzed on the gel, are indicated. **B)** Samples of Rat 4 nuclear extract (3 ug) were incubated in the absence (-) or presence of 1ul p50 antibody (sc-114) (α p50) for 60 min and subjected to EMSA using an NF-κB oligonucleotide, as probe. Film was exposed for 6 h. **C)** Samples of Rat 4 nuclear extract (3 ug) were incubated in the absence (-) or presence of 1 ul p65 antibody sc-372 (α p65) for 60 min and then subjected to EMSA using an NF-κB oligonucleotide, as probe. Film was exposed for 5 days.

Fig. 2. Nuclear extracts of mammary glands from DMBA-treated S-D rats display high NF-κB/Rel binding activity prior to tumor formation. **A and B)** NF-κB EMSA. EMSA was performed using samples (5 ug) of nuclear extracts from mammary glands of rats treated with DMBA (A) or representative control 6 hour control rats (B), and an NF-κB oligonucleotide, as probe. In each panel, a sample (5 ug) of nuclear extract from a DMBA-treated rat mammary tumor (Tumor) was run to allow for comparison between gels, and to serve as a normalization for intensity of binding observed in the various samples. Densitometry analysis was performed to compare the relative level of NF-κB/Rel binding in nuclear extracts from mammary glands from the control or DMBA-treated animals, and intensity values are given as percent of tumor binding intensity (where the Tumor intensity was set at 100%). Asterisks (*) denote animals whose binding intensity were at least one standard deviation above the average intensity of the control animals. **C)** PU.1 EMSA. EMSA was performed using samples (5 ug) of nuclear extracts from mammary glands of the 3-week DMBA-treated rats (46-50) with the PU.1 oligonucleotide, as probe. As a positive control for binding, nuclear extracts from WEHI 231 B cells were analyzed on the same gel. **D)** TCF-1 EMSA. Samples (5 ug) of nuclear extracts from mammary glands of the indicated 3-week DMBA-treated rats and a DMBA-induced mammary tumor were subjected to EMSA with the TCF-1 oligonucleotide, as probe (28). As a positive control for binding,

nuclear extracts (5 ug) from Jurkat T cells and from an activated T-cell hybridoma were analyzed on the same gel.

Fig. 3. Carcinogen-transformed D3-1 and BP-1 cells display higher constitutive levels of functional NF-κB than the parental MCF-10F cells. **A)** Comparison of lines. The MCF-10F cells (10F) and BP-1 cells were transiently transfected by lipofection, in triplicate or duplicate, respectively with 2 ug E8 or mutE8 reporter construct. Alternatively, D3-1 cells were transfected, in duplicate, using 20ug of either E8 or mutE8 by the calcium phosphate method. After 24 hours (for lipofectamine) or 72 hours (for calcium phosphate), extracts were prepared, normalized for protein, and assayed for CAT activity. The values for E8 CAT activity are represented as fold induction over mutE8 CAT activity which was set at 1.0 for each cell line. Shown is the representative data from a minimum of 2 experiments. (Inset) Equal amounts (5 ug) of nuclear extracts from exponentially growing parental MCF-10F cells or transformed D3-1 or BP-1 cells were subjected to EMSA with a radiolabeled oligonucleotide NF-κB element as probe. A representative experiment of two independent assays is shown. **B)** Activity is inhibited with IκB-α expression. D3-1 cells were transiently transfected, in duplicate, with 15 ug of E8 reporter construct and 0, 1, 3, or 7 ug of PMT2T IκB-α expression vector, in the presence of 2.5 ug TK-luciferase construct for normalization of transfection efficiency. The total amount of plasmid DNA transfected in each sample was adjusted to 25 ug by addition of pBlueScript+ plasmid DNA. Alternatively 25 ug of pBlueScript DNA was used alone. Lysates were prepared after 72 hours and analyzed as described previously (12).

Fig. 4. Supershift analysis reveals classical NF-κB subunits in the binding complex.
A) Supershift analysis of MCF10F cells. Following a 30 min incubation of nuclear extracts (5 ug) with the probe, 1 ul of antibody against either the p50 (kindly provided by K. LeClair) or p65 (sc-109) was added as indicated, and the reaction incubated for an additional 1 hr and subjected to EMSA as above. **B)** Supershift analysis of D3-1 cells. Following a 30 min incubation of nuclear extracts (5 ug) with the probe, 1 ul of antibody against the p50 (sc-114), p65 (#1226, kindly provided by N. Rice) or c-Rel (sc-070) was added as indicated, incubated and subjected to EMSA.

Fig. 5. IκB-α protein in D3-1 and BP-1 cells has a shorter half life than in parental MCF-10F cells. **A)** MCF-10F, D3-1 and BP-1 cells were incubated in the absence or presence of 20 ug/ml emetine for the indicated periods of time. Cytoplasmic extracts (50 ug protein/lane) were separated by electrophoresis in a 10% polyacrylamide-SDS gel, and subjected to immunoblot analysis for IκB-α protein using SC-371 antibody. These blots are representative of two experiments. **B)** The immunoblot for IκB-α protein in part A was quantitated by densitometry, and the data plotted as percent of the original protein value in untreated control cells (0 hr). The decay curves were extrapolated by using an exponential best fit analysis.

Fig. 6. Ectopic expression of p65 in MCF-10F cells increases c-Myc oncoprotein levels. Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4 μg *pEVRF-p65* plus 20 μg *pcDNA3* plasmid or with 24 μg *pcDNA3* plasmid DNA with 30 μl FUGENE transfection reagent. After 48 h, cells were rinsed with cold PBS, and WCEs prepared. Samples (40 μg) were subjected to electrophoresis and immunoblot analysis for c-Myc (786-4, anti-c-Myc antibody), and β-actin (AC-15, Sigma).

Fig. 7. Dysregulated NF-κB/Rel expression in 184A1 immortalized HMECs. **A)** 184A1 cells display increased NF-κB binding in quiescence. EMSA was performed with nuclear extracts (5 ug) from finite life-span 184 and fully immortal 184A1 cells following G_0 synchronization upon blockage of EGF receptor signal transduction for 48 hours (G0), or during exponential growth (CYC). Two distinct NF-κB binding complexes were detected. **B)** Supershift EMSA reveals classical NF-κB in the binding complex of 184A1 cells. Supershift analysis was performed on nuclear extracts (5 ug) harvested from fully immortal 184A1 HMECs in exponential growth, or made quiescent following blockage of EGF receptor signal transduction for 48 hours. Following a 30 min incubation of nuclear extracts with the probe, 1 ul of antibody against p50 (sc-114) or p65 (#1226) was added as indicated, and the reaction incubated for an additional 1 hr and subjected to EMSA, as above. The upper binding complex is composed of p65/p50 subunits, and lower binding complex is a p50/p50 homodimer. Position of a nonspecific band is indicated by an asterisk (*).

Fig. 8. Transient transfection analysis was performed on the in both the G0 and exponential cycling states. Twenty-four hours after plating (110,000 cells/sample), finite life-span 184 and fully immortal 184 A1 cells were transfected, in duplicate, with equivalent amounts of either the E8 or mutE8 plasmid using Cytofectin reagent. Total amount of DNA was normalized to be either 1 or 1.5 ug (equivalent within each experiment) using pBlueScript. Cells were washed in PBS after 5 hours, and fresh media was added. After 24 hours, cells were either given fresh normal medium (CYCLING), or medium minus EGF plus MAb 225 (G0). Cells were harvested 24 hours later in reporter lysis buffer, and assayed for CAT activity. The values for E8 CAT activity are represented as fold induction over mutE8 CAT activity which was set at 1.0 and were normalized for protein levels. Shown is the representative data from a minimum of 2 experiments.

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A)
Band 2
Band 1
R1
R2
R3
R4
R5
c-Rel/p50
p50/p50
B)
Band 1
−
+ α p50
C)
Band 2
Band 1
−
+ α p65

Figure 1 A, B, C

A)

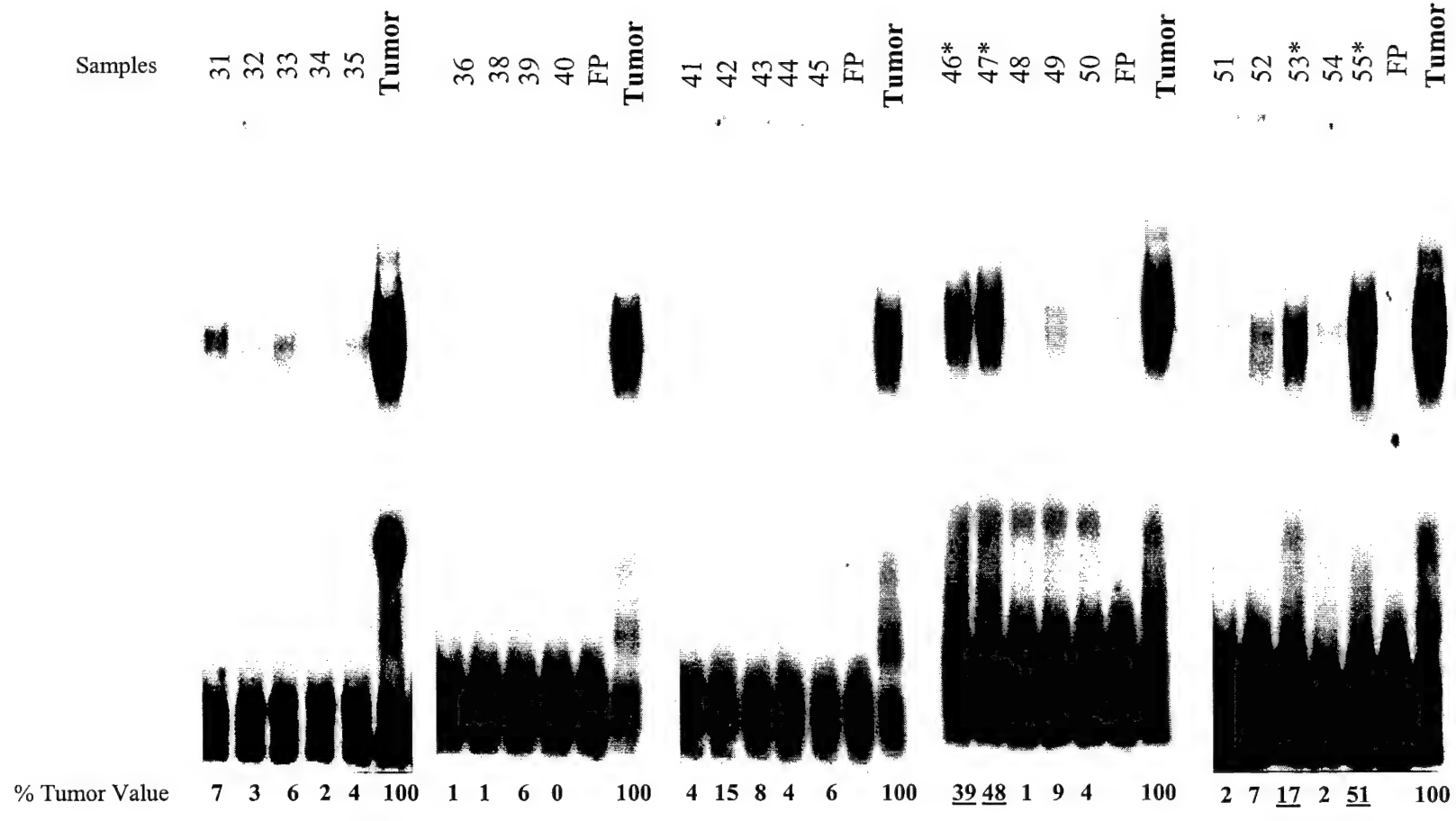
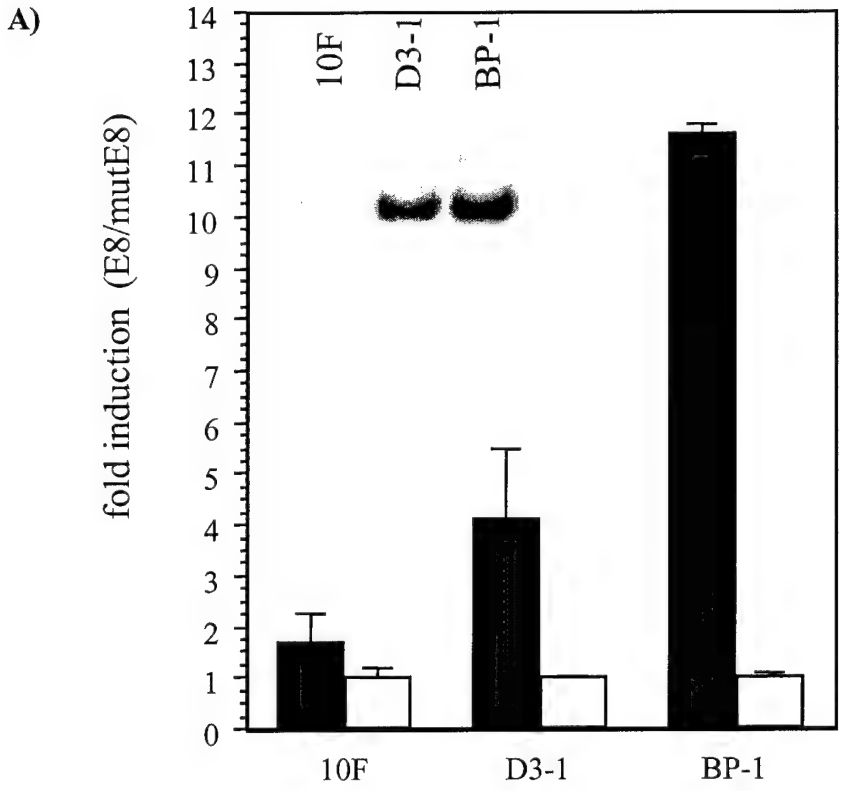

Samples
31 32 33 34 35 Tumor
36 38 39 40 FP Tumor
41 42 43 44 45 FP Tumor
46* 47* 48 49 50 FP Tumor
51 52 53* 54 55* FP Tumor
% Tumor Value
7 3 6 2 4 100
1 1 6 0 100
4 15 8 4 6 100
39 48 1 9 4 100
2 7 17 2 51 100
6 Hours
24 Hours
1 Week
3 Weeks
9 Weeks

Figure 2A

B)
Samples
1
2
3
4
Tumor
% Tumor Value
2
4
3
11
100
C)
PU.1
WEHI 231
46
47
48
49
50
D)
TCF
Jurkat T-cell
T-cell Hybridoma
Tumor
46
47
49
50

Figure 2 B, C, D

Figure 3


A)
fold induction (E8/mutE8)
10F
D3-1
BP-1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
10F
D3-1
BP-1

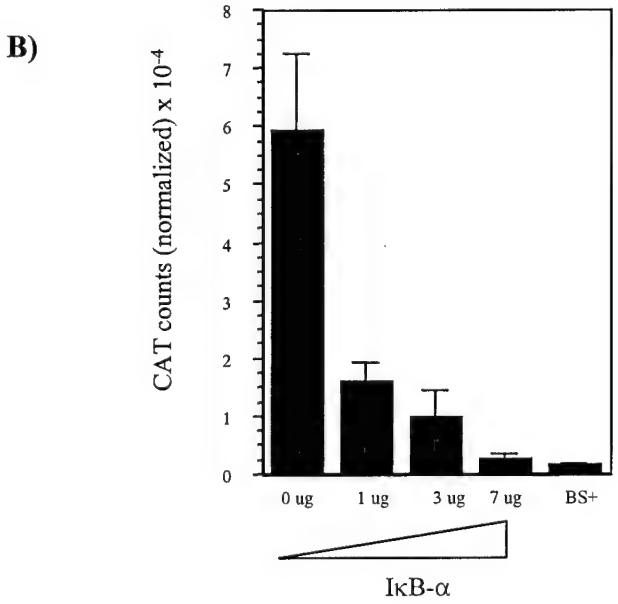
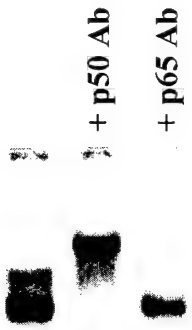

B)
CAT counts (normalized) x 10-4
0
1
2
3
4
5
6
7
8
0 ug
1 ug
3 ug
7 ug
BS+
IκB-α

Figure 4

A)



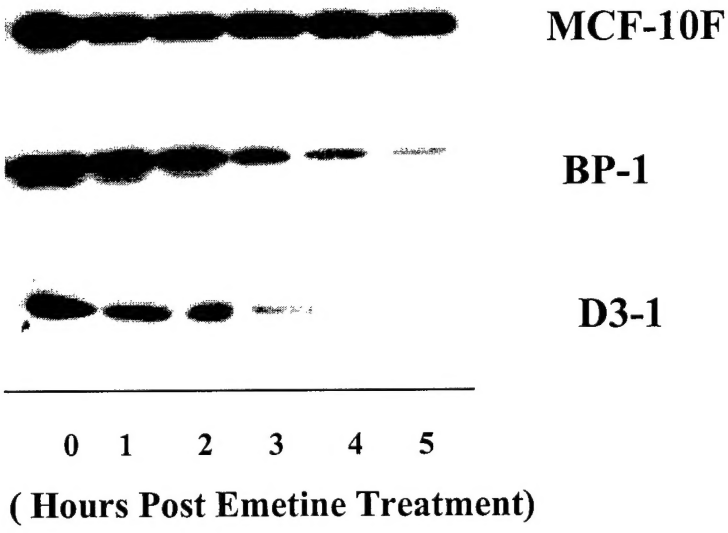
MCF-10F

B)

D3-1

Figure 5

A)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BP-1
D3-1
0 1 2 3 4 5
(Hours Post Emetine Treatment)

B)

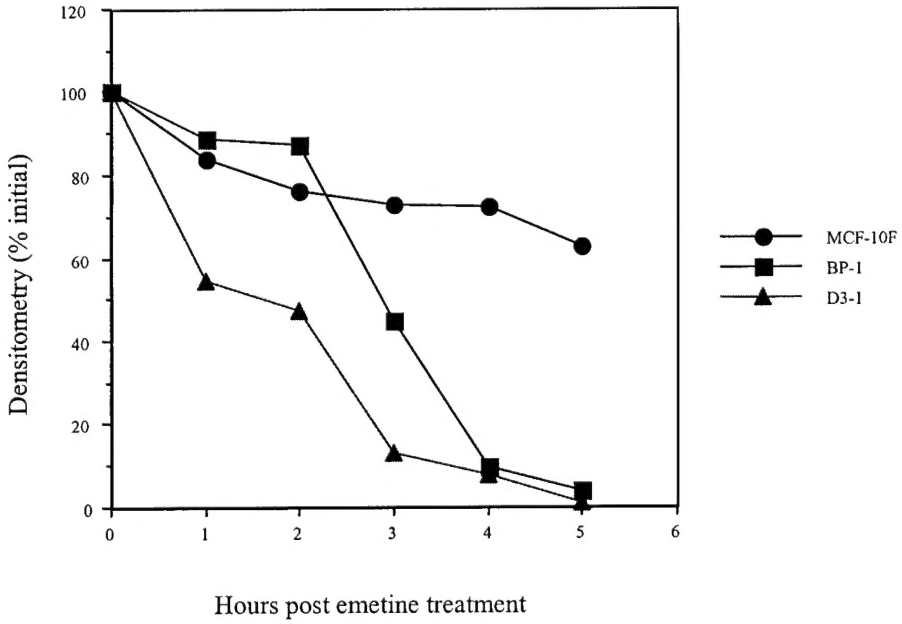
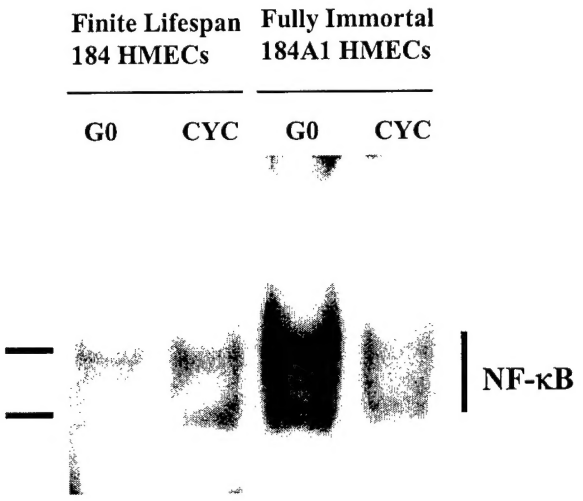

120
100
80
60
40
20
0
Densitometry (% initial)
0 1 2 3 4 5 6
Hours post emetine treatment
MCF-10F
BP-1
D3-1

Figure 6

A)


Finite Lifespan
184 HMECs
Fully Immortal
184A1 HMECs
G0
CYC
G0
CYC
NF-κB

B)

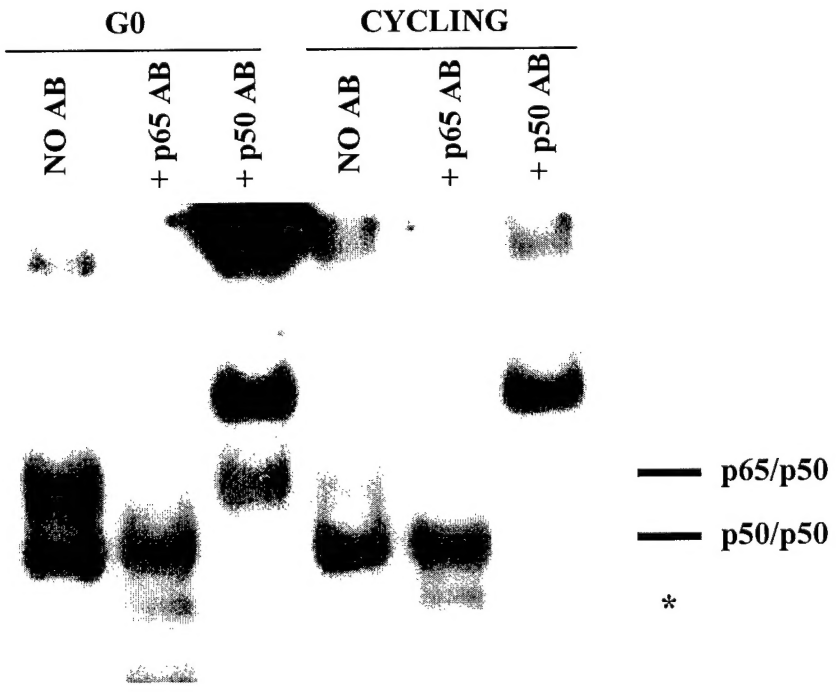
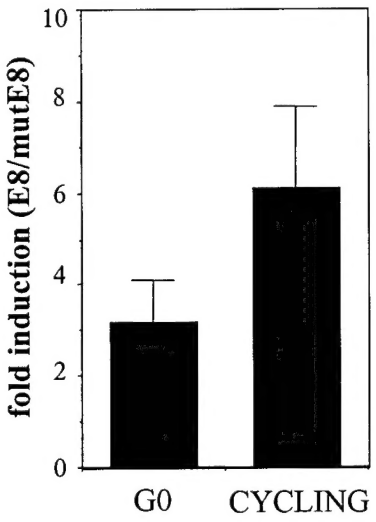
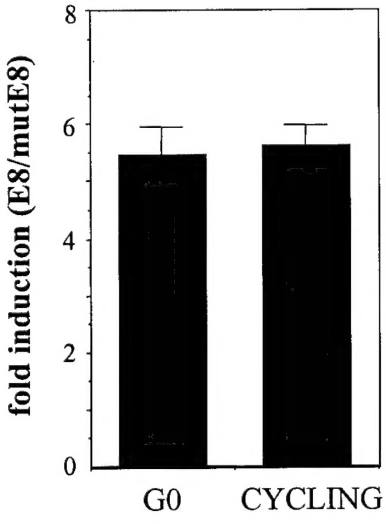

G0
CYCLING
NO AB
+ p65 AB
+ p50 AB
NO AB
+ p65 AB
+ p50 AB
p65/p50
p50/p50
*

Figure 7

Figure 8



Finite Lifespan 184 HMECs



Fully Immortal 184A1 HMECs